HORIZONTAL CHROMOSOMAL GENE TRANSFER AMONG STRAINS OF *Xanthomonas axonopodis* pv. *vesicatoria* BY CONJUGATION

By

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By

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Transfer of chromosomal DNA among strains of *Xanthomonas axonopodis* pv. *vesicatoria* by conjugation was investigated *in vitro* and *in planta*. Conjugants (10^{-8} per donor cell) were detected on media containing copper, rifamycin and nalidixic acid. Plasmid profiles and total genomic DNA profiles determined by endonuclease digestion with *Spe*I and Pulsed-Field Gel Electrophoresis (PFGE) of donor, recipient and conjugants confirmed chromosomal gene transfer during conjugation *in vitro*. A unique chromosomal copper-resistance gene-cluster from a strain of *X. axonopodis* pv. *vesicatoria*, which was used as a donor was cloned to use as a genetic marker on the chromosome of the strain. A Tn*5* chromosomal marker of another donor strain was transferred to a recipient that was naturally sensitive to kanamycin. The copper resistance genes and Tn*5* sequences were identified on restricted DNA fragments of conjugants and donor but not on recipient by Southern hybridi-

zation. The optimal incubation period for conjugal transfer of the copper resistance genes and Tn*5* sequences was 3 days *in vitro.*

The occurrence of chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria* by conjugation was determined in leaves of pepper plants. Depending upon donor strain conjugants were detected on media containing copper, rifamycin, nalidixic acid and chlorothalonil, or kanamycin, rifamycin, nalidixic acid and chlorothalonil. Chromosomal sequences associated with kanamycin resistance (Tn*5*), copper resistance genes (*cop*), hypersensitive reaction and pathogencity (*hrp*) and pigmentation genes (*pig*) were transferred from donor strain to recipient strain of the bacterium. The optimal incubation period for conjugal transfer of chromosomal genes *in planta* was 48 hr (until lesion formation starts in inoculated leaf tissue). The frequency of chromosomal gene transfer was more than 100 times greater in pepper leaves than *in vitro*.

Differential rates of transfer of *hrp* and *pig* occurred in pepper plants. A kanamycin resistant donor strain was mated with both *hrp*$^-$ and *pig*$^-$ recipient strains of the bacterium for 48 hr. Conjugants were resistant to kanamycin, rifamycin, and nalidixic acid. Horizontal transfer of *hrp* genes occurred in 5% of 400 conjugants tested. Horizontal transfer of *pig* genes occurred in 90% of 400 conjugants tested. Tn*5* and *pig* genes were located on the same *Spe*I restricted fragment separated by PFGE. On the other hand, Tn*5* and *hrp* genes were located on different *Spe*I restricted fragments separated by PFGE.

Transfer genes (*tra*) which are responsible for DNA transfer during conjugation were cloned from a donor strain. The *tra* genes were located in a library of DNA of the strain in pLAFR3. The *tra* genes mobilized movement

of pLAFR3 between strains of *X. axonopodis* pv. *vesicatoria*. Horizontal transfer of chromosomal genes *cop*, *pig*, *hrp*, and Tn*5* sequences from a donor to a recipient strain of *X. axonopodis* pv. *vesicatoria*, and its occurrence *in planta* in an inducible manner indicates that horizontal chromosomal gene transfer is entirely possible in nature. This type of gene transfer may explain the presence of great diversity among strains of the bacterium in nature in terms of restriction fragment length polymorphism, and may also explain the apparent horizontal transfer of *hrp* gene sequences among pathovars of *Xanthomonas*.

CHAPTER 1
LITERATURE REVIEW

Introduction

Xanthomonas axonopodis pv. *vesicatoria* (= *X. campestris* pv. *vesicatoria*) causes the bacterial spot disease of tomato (*Lycopersicon spp.*) and pepper (*Capsicum spp.*) wherever these crops are grown in the world (Stall, 1993). High rainfall and high temperature are favorable conditions for severe disease which reduces growth and yields compared to healty plants (Stall, 1993). Necrotic spots on leaves, stems, peduncles, and fruits are distinctive symptoms of the disease (Cox et al., 1956; Jones, 1991), and lesions on fruits result in loss of marketability (Stall et al., 1994).

General Characteristics of *Xanthomonas*

Bacteria in the genus *Xanthomonas* Dowson 1939 are Gram-negative rods and most of them form yellow mucoid smooth colonies. Cells are usually motile by means of one polar flagellum (Vauterin et al., 1995). *Xanthomonas spp.* occurs worldwide and causes plant diseases in over 124 monocotyledonous and 268 dicotyledonous plant species (Hayward, 1993; Leyns et al., 1984).

The genus, *Xanthomonas,* has a great deal of genetic variation in nature, and comprises 20 DNA homology groups which are considered genomic species. All of the strains in the same species were related at an average level of homology of 77%. The average levels of DNA hybridization between strains

belonging to different *Xanthomonas* DNA homology groups were less than 40%. Two major classes of DNA homology could be differentiated among pathovars of *Xanthomonas campestris.* One class includes genomically almost unrelated strains; a second class includes genomically highly related strains exhibiting levels of DNA homology of more than 80% (Vauterin et al., 1995).

Comparisons of genomic fingerprinting and RFLP analyses have also shown significant genomic diversity among and within different pathovars of *Xanthomonas campestris* (Egel et al., 1991; Lazo and Gabriel, 1987; Stall et al., 1994). The *hrp* gene (hypersensitive reaction and pathogenicity) sequences of *X. axonopodis* pv. *vesicatoria* were conserved among a large number of pathovars of *X. campestris*, as well as in related *Xanthomonas spp.* (Leite et al., 1994).

Xanthomonas axonopodis pv. *vesicatoria*

Bacterial spot of pepper (*Capsicum spp.*) and tomato (*Lycopersicon spp.*) causes a severe disease in climates where high rainfall and high temperatures occur concomitantly (Stall, 1993). The disease on young plants causes more yield reduction than on mature plants (Pohronezny and Volin, 1983; Stall, 1993).

Numerous strains of the organism occur that have specific pathogenicities. Strains pathogenic to tomato and pepper, to pepper only, and to tomato only may occur in various regions. Specific interactions appear to be inherited in both the pathogen and the plant in a gene-for-gene manner (Stall, 1993). Avirulence genes in the bacterium are involved in the host-specificities as well as the cultivar specificities (Minsavage et al., 1990b; Stall, 1993). Strains of

X. a. pv. *vesicatoria* resistant to copper and streptomycin are commonly selected in fields sprayed with those materials (Marco and Stall, 1983; Stall and Thayer, 1962).

Strains have a great diversity in terms of restriction fragment length polymorphism (Cooksey and Graham, 1989; Stall et al., 1994). The bacterium has also great diversity in plasmid content, and plasmids vary in size from 3 to 300 kb among a population of nearly 500 strains. Thirteen size-classes of plasmids were differentiated and 71 different profiles occurred among the strains (Canteros, 1990; Stall, 1993). Most of the avirulence genes (Minsavage et al., 1990a) and genes for resistance to copper and streptomycin are located on plasmids (Minsavage et al., 1990b; Stall et al., 1986). The plasmids that contain copper resistance genes are self-transmissible (Stall et al., 1986). The horizontal transfer of plasmids among strains of *X. axonopodis* pv. *vesicatoria* occur in nature (Canteros et al., 1995).

Two groups of strains were distinguished within *X. campestris* pv. *vesicatoria* based on physiology. Strains of group B are pectolytic and amylolytic; strains of group A are negative for those characteristics (Stall et al., 1994). Recently, based upon DNA-DNA hybridization study of *Xanthomonas* pathovars, group A strains were reclassified as *X. axonopodis* pv. *vesicatoria* and group B strains were reclassified as *X. vesicatoria* (Vauterin et al., 1995).

Copper Resistance

Copper compounds have been used for several decades in the control of bacterial and fungal plant pathogens, but copper-resistant plant pathogens

have only recently been reported (Adaskaveg and Hine, 1985; Bender and Cooksey, 1986; Lee et al., 1994; Marco and Stall, 1983; Sundin et al., 1989).

Copper is an essential trace element required for several bacterial enzymes, particularly oxidation-reduction enzymes (Cass and Hill, 1980; Lim and Cooksey, 1993). However, excess copper is toxic to cells. Most determinants of copper resistance in phytopathogenic bacteria (Bender and Cooksey, 1986; Bender et al., 1990; Cooksey, 1987; Cooksey, 1990a; Cooksey, 1990b; Stall et al., 1986; Voloudakis et al., 1993), and some other bacteria including *Escherichia coli* (Tetaz and Luke, 1983) and *Mycobacterium scrofulaceum* (Erardi et al., 1987), are plasmid-encoded. Chromosomal copper resistance genes were cloned from *X. arboricola* pv. *juglandis* (Lee et al., 1994).

Copper resistance genes in *X. axonopodis* pv. *vesicatoria* from Florida and Oklahoma are located on 200 and 188-kb self-transmissible plasmids (Bender et al., 1990; Stall et al., 1986). Copper resistance genes in *X. axonopodis* pv. *vesicatoria* strains isolated from tomato plants and seeds in California are located on a non-self-transmissible plasmid approximately 100 kb in size (Cooksey et al., 1990).

The genetic and molecular bases of copper resistance of *Pseudomonas syringae* pv. *tomato* and *E. coli* have been well studied (Bender and Cooksey, 1987; Brown et al., 1992; Cooksey, 1990a; Mellano and Cooksey, 1988b; Silver and Walderhaug, 1992). Copper resistance genes from *P. syringae* pv. *tomato* were located on 35-kb mobilizable plasmid (pPT23D) (Bender and Cooksey, 1987; Mellano and Cooksey, 1988b). The copper resistance genes are pPT23D are organized as an operon (*cop*) consisting of four genes (*copABCD*)

under the control of a copper-inducible promoter (Mellano and Cooksey, 1988a; Mellano and Cooksey, 1988b). Two regulatory genes (*copRS*) with similarities to other two-component regulators lie 3' to *copABCD*. The regulatory genes are required for copper-inducible expression of the *cop* promoter, but they are transcribed separately by a constitutive promoter 5' to *copR* (Mills et al., 1993). *CopA* and *CopC* are periplasmic copper-binding proteins, and *CopB* is an outer membrane protein. The proteins function in the periplasm and outer membrane in copper sequestration , which may prevent toxic levels of copper from entering the cytoplasm (Cha and Cooksey, 1991). *CopD* functions in copper transport (Cha and Cooksey, 1993).

The resistance determinants from *X. campestris* have the same general *copABCD* structure as *P. syringae*, but with some differences in gene size and spacing, and considerable divergence at the sequence level. There also is no structural or functional evidence for *copRS* homologs in *X. campestris* (Lee et al., 1994).

Bacterial Conjugation

Three processes of gene transfer are known in bacteria; transformation, transduction, and conjugation (Provence and Curtiss, 1994). Bacterial conjugation was discovered and described as a process in which genetic material from one bacterial strain to another is dependant upon cellular contact between members of two bacterial strains (Curtiss, 1969; Lederberg and Tatum, 1946). The *Escherichia coli* strain, K12, carries a conjugative plasmid called F and a factor for antibiotic resistance. The bacterial conjugation system mediated by

F sex factor was subjected to detailed genetic and molecular analysis (Iphen-Ihler and Minkley, 1986; Willets and Skurray, 1987). The F-like plasmids, many of which carry determinants for antibiotic resistance and for toxin and hemolysin production, were grouped together on the basis of the sex pili that were elaborated by hosts carrying them. These pili which are morphologically and serologically similar to those encoded by F rendered the host susceptible to F- or "male-specific" phages (Ippen-Ihler and Skurray, 1993). The plasmids, also classified on the basis of incompatibility (Inc) as determined by two plasmids in the same Inc group, could not both stably coexist in the same host cell (Datta, 1975).

tra Genes

The genes involved in F-directed conjugation are located within the 34-kb *tra* (transfer) region on the F plasmid. Sequence analysis of the *tra* genes from F-like plasmids has allowed the comparison of a number of different alleles (Ippen-Ihler and Skurray, 1993).

The functions of products of genes *tra A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *K*, *L*, *M*, *N*, *S*, *T*, *U*, *V*, *W* were deduced from the characteristics of F *lac* transfer deficient or surface-exclusion-deficient derivatives carrying mutations that affect individual *tra* cistrons (Willets and Skurray, 1980). The protein product and DNA sequence analyses revealed the existence of 16 additional transfer region loci (*tra P*, *Q*, *R*, *Y*, *X*, and *trb A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *artA*) were determined that were not represented in earlier mutant collections (Ippen-Ihler and Skurray, 1993).

Transfer loci of F plasmid involved in gene regulation and expression,

and promoters, P_M and P_J which initiate individual transcripts for *traM* and *traJ* are located near the beginning of the *tra* region (Dempsey, 1987; Dempsey, 1989; Gaffney et al., 1983; Thompson and Taylor, 1982). However, the majority of the *tra* region loci are expressed from P_Y and activation of P_Y requires TraJ, and the host protein, SfrA (Mullineaux and Willetts, 1985; Silverman et al., 1991a; Silverman et al., 1991b). TraJ product is the positive regulator for expression of the majority of downstream *tra* genes, and expression of *tra* genes that are repressed by *finO* and *finP*. The transcript initiated from the *traJ* promoter includes an untranslated leader sequence that can interact with FinP. The FinP repressor is a small RNA transcript, complementary to the *traJ* mRNA leader sequence and the translational start site for *traJ*. FinP is expressed from a promoter located near the beginning of the *traJ* open reading frame but on the antisense strand. *finP* RNA inhibits *traJ* expression by annealing to its complementary sequence on the *traJ* mRNA. *finP* genes are plasmid specific (Ippen-Ihler and Skurray, 1993).

Expression of the transfer region of an F-like plasmid leads to the elaboration of long, flexible, plasmid-specific filaments (pili). Usually only one or two F pili are visible per donor cell and are hollow, cylindrical, 8 nm in diameter with an axial hole of 2 nm, and extend 1 to 2 μm from the cell surface (Marvin and Folkhard, 1986; Paranchych and Frost, 1988; Willetts and Skurray, 1980). F pili are assembled from an inner membrane pool of F-pilin subunits (Moore et al., 1981).

The assembly process is reversible (Novonty and Fives-Taylor, 1974; Sowa et al., 1983). The F pilus acts as an external receptor, and once phages and recipient cells attach to the pilus, they can be brought to the cell surface

when the pilin subunits disassemble and the pilus retracts (Iphen-Ihler and Skurray, 1993).

The mixtures of piliated F donors and recipient (F^-) cells typically aggregate into clusters of cells in surface contact (Achtman, 1975). Treatment with 0.01% sodium dodecyl sulphate (SDS) to disrupt F pili can prevent the aggregates from forming but does not prevent DNA transfer once it has been started (Achtman et al., 1978; Panicker and Minkley, 1985). Although there is some evidence that DNA transfer can occur through extended F-pilus filaments (Harrington and Rogerson, 1990; Ou and Anderson, 1970), transfer typically occurs between cells held closely together (Iphen-Ihler and Skurray, 1993).

The formation of wall-to-wall contacts between donor and recipient cells was observed by video microscopy. These contacts were correlated with conjugative junctions between the outer membrane of the cells using transmission electron microscopy (Durrenberger et al., 1991).

Mobilizable Plasmids in Gram Negative Bacteria

Most conjugation systems in Gram Negative bacteria are encoded by plasmids and more than 25 different groups of plasmids have been defined on the basis of incompatibility (Inc) properties; many of these specify distinct transfer systems (Courturier et al., 1988). Many self-transmissible plasmids originally isolated in a species of *Enterobacteriaceae* could be transferred to other members of this family. However, only certain plasmids could be transferred to other members of this family on to *Pseudomonas* species. Those were termed "broad host range plasmids," and those confined to the

Enterobacteriaceae were "narrow host range plasmids" (Guiney, 1993). Although some of the plasmids naturally found in either the *Enterobacteriaceae* or *Pseudomonas* can mediate transfer to heterologous hosts, they can not replicate in the hosts (Finger and Krishnapillai, 1980; Guiney, 1982; Krishnapillai, 1988; Tardiff and Grant, 1983). Plasmids isolated from *Pseudomonas* are divided into broad and narrow host range depending on their ability to be transferred and maintained in *Escherichia coli* (Datta and Hedges, 1972; Jacob et al., 1977; Jacoby and Shapiro, 1977).

A careful analysis of the host range of self-transmissible plasmids revealed that the property depended on many traits, including the conjugation system, the replication and maintenance functions, and the ability of plasmid-encoded selective markers to be expressed in the new host (Guiney, 1993).

A general approach has been to separate the conjugation system from the vegetative replication and maintenance region. The host range of each of these systems was tested for the *E. coli* fertility plasmid F (IncF1) and compared to the promiscuous IncP plasmid RK2 (Guiney, 1982). RK2 was 10^4 times more efficient than F at mobilizing a test plasmid from *E. coli* to *Pseudomonas aeruginosa*, while both systems were equally effective in transfers between *E. coli* strains (Guiney, 1993).

The promiscuous IncP plasmids can replicate in many species of the α, β, and γ subgroups of the purple bacteria (Thomas and Helinski, 1989). However, they cannot replicate in the δ subgroups (*Myxococcus*) (Breton et al., 1985), in *Cyanobacteria* (Wolk et al., 1984), and the Gram Negative anaerobe *Bacteroides fragilis* (Guiney et al., 1984). IncP plasmids are potentially useful tools for genetic studies of chromosomal loci in a wide variety of Gram Nega-

tive bacteria. For example, the ability of the F plasmid to form Hfr strains was instrumental in mapping the *E. coli* chromosome and considerable interest has focused on the ability of IncP plasmids to mobilize chromosomal genes in a number of Gram Negative hosts (Haas and Reimmann, 1989).

DNA Processing During Plasmid Transfer Between Gram Negative Bacteria

Gram Negative conjugation can be divided operationally into two stages. The first involves the formation of a specific bridge between the plasmid-containing donor bacterium and the recipient cell, which are brought into contact by the extracellular conjugative pilus. The second stage is nicking of the plasmid at the specific origin of transfer (*oriT*) site and unwinding of the duplex by one or more DNA helicases and the transfer of the open DNA strand to the recipient cell. The transferred DNA is then circularized by a mechanism involving interactions at the *oriT* sequence. Such single-stranded (ss) DNA transfer is normally associated with conjugative DNA synthesis, generating a replacement strand in the donor cell and a complementary strand in the recipient (Wilkins and Lanka, 1993).

The *oriT* site of several different conjugative plasmids is located at or near one end of the DNA segment carrying the defined *tra* genes. This arrangement is found on F-like plasmids (Ippen-Ihler and Minkley, 1986) and on IncI1 (Rees et al., 1987). The conjugative plasmids are transferred in a preferred direction from *oriT*, and *tra* genes enter the recipient cell last (Al-Doori et al., 1982; Grinter, 1981; Howland and Wilkins, 1988). The cluster of mobility genes of ColE1 is transferred late during mobilization (Boyd and Sherratt,

1986; Boyd et al., 1989). The late transfer of the *tra* genes may confer some advantage, possibily to delay their expression in the newly infected recipient cell until completion of DNA transport and circularization and expression of plasmid genes in the recipient actively terminates the conjugation cycle, but the timing of this termination event relative to a cycle of DNA transfer has not been established (Wilkins and Lanka, 1993).

The DNA strand of the donor is transferred to the recipient cell in the 5' to 3' orientation, and the complementary strand is retained in the donor (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Vapnek and Rupp, 1970; Vapnek et al., 1971).

A particularly elusive aspect of conjugation is the nature of the bridge or pore supporting DNA transfer. The conjugative pilus is necessary for the formation of intercellular contact, but there is no conclusive evidence that DNA undergoing transfer is associated with an extended pilus. DNA transfer occurs between cells that are brought into surface-surface contact by pilus retraction and subsequently stabilized as aggregates (Wilkins and Lanka, 1993). The pore for DNA transport allows no general mixing of the cytoplasmic contents of conjugating bacteria (Rees and Wilkins, 1989).

The transferred F strand enters the cytoplasm of the recipient cell without detectable association with any *tra* gene product. However, the transferred DNA of some other plasmids, such as ColIb and RP4, is accompanied into the recipient by specific *tra* products called DNA escort proteins (Wilkins and Lanka, 1993).

Mobilization of Chromosomes

Naturally occurring conjugative plasmids transfer themselves, and in many cases they can also transfer chromosomal DNA. Chromosomal mobilization can happen in two ways. In the first, designated in transmobilization the conjugative plasmid and the mobilized DNA remain physically separated throughout the mobilization process. In the second, designated *in cis* mobilization proceeds via the formation of a cointegrate between the conjugative plasmid and the replicon to be mobilized, and the essential feature of this type of mobilization is that the transfer-proficient plasmid recombines with the other replicon, resulting in a covalent link between the two replicons (Reimmann and Haas, 1993).

Conjugative chromosome transfer systems were established in many bacterial genera (*Pseudomonas*, *Streptomyces*, *Rhizobium*, etc.) and permitted the construction of chromosome maps.

Stable integration of a conjugative plasmid into the bacterial chromosome gives rise to Hfr (High frequency of recombination) donor strains and chromosome transfer proceeds unidirectionally from the site of plasmid integration (Haas and Reimmann, 1989; Hayes, 1968) and proximal chromosomal markers are transferred at frequencies of 10^{-1} to about 10^{-4} per donor, depending on the efficiency of conjugation, i.e., on the type of conjugative plasmid integrated (Reimman and Haas, 1993). Hfr strains are used to map chromosomal markers according to time of entry by the classical technique of interrupted mating (Hayes, 1968; Low, 1987). The classical circular chromosome maps of *E. coli* , *S. typhimurium*, *Pseudomonas aeruginosa*, and *P. putida* have all been calibrated in time units as determined by Hfr crosses (Reimman and Haas, 1993).

Time-of-entry data have helped to order markers in other Gram Negative bacteria, including *Citrobacter freundii* (De Graaff et al., 1974), *Erwinia amylovora* (Chatterjee and Starr, 1973), *P. syringae* (Nordeen and Holloway, 1990), and *Methylobacillus flagellatum* (Tsygankow et al., 1990).

The F plasmid of *E. coli* integrates spontaneously into the chromosome, and in many cases, remains there stably (Curtiss and Stallions, 1969; Hayes, 1968). *E. coli* strains carrying an autonomous F plasmid are chromosome donors, although markers are transferred at much (about 10^{-5}-fold) lower frequencies than those observed in Hfr donors in the F^+ population. However, 85% of the recombinants arise by another mechanism either by transient and unstable integration of F into the chromosome or by some kind of mobilization *in trans* (Reimmann and Haas, 1993).

The natural occurrence of an *oriT* in the chromosomes of Gram Negative bacteria has not been demonstrated; however, an *oriT* can be introduced into the chromosome by artificial transposons such as Tn*5*-Mob (Reimmann and Haas, 1993).

The frequency of recombination between the linear incoming chromosome fragment and the resident chromosome may exceed 50% in *Rec*$^+$ recipients (Hayes, 1968; Smith, 1988; Smith, 1991), because the incoming linear DNA is an excellent substrate for the *Rec*BCD enzyme (Smith, 1988; Smith, 1991).

Several R plasmids whose conjugative functions are normally repressed have been mutated to give derepressed (drd) variants, and R100 drd-19 in *E. coli* (Pearce and Meynell, 1968), R144 drd-3 in *Klebsiella pneumoniae* (Dixon et al., 1975), and R100 drd-56 in *Erwinia amylovora* (Chatterjee and Starr,

1980), have been shown to have chromosome-mobilizing properties (Cma). Indirect experimental evidence for the importance of recombination comes from conjugative plasmids that have been genetically manipulated to carry active transposable elements or DNA fragments having homology with chromosomal regions. Transposons Tn*501* which belong to the Tn*3* family, enhance the Cma of IncP plasmids in *Rhodobacter sphaeroides* (Pemberton and Bowen, 1981). Transposon-facilitated recombination (Tfr) donors (Tn*1* or Tn*5* present in their chromosome and conjugative plasmid) facilitate chromosome transfer. Tfr donors have been constructed in *P. aeruginosa* (Krishnapillai et al., 1981); *Vibrio cholerae* (Johnson and Romig, 1979), a marine *Vibrio* strain (Ichige et al., 1989), *Agrobacterium tumefaciens* (Hooykaas et al., 1982; Pischl and Farrand, 1983), and in a Gram Positive bacterium, *Staphylococcus aureus* (Stout and Iandolo, 1990).

Insertions of chromosomal fragments into IncP plasmid *in vitro* or *in vivo* produces R' plasmids that result in oriented chromosome transfer in *Escherichia coli* (Barth, 1979), *Pseudomonas aeruginosa* (Holloway, 1978), *Proteus morganii* (Beck et al., 1982), and *Rhizobium meliloti* (Julliot and Boistard, 1979).

The principal application of sex plasmids is classical chromosome mapping. Genetic circularity has also been demonstrated for the chromosomes of several other Gram Negative bacteria including *Agrobacterium tumefaciens* (Hooykaas et al., 1982; Miller et al., 1986), *Rhodopseudomonas capsulata* (Willison et al., 1985), *Proteus morganii* (Beck et al., 1982), *Acinetobacter calcoaceticus* (Vivian, 1987), *Rhizobium leguminasarum*, and *Rhizobium*

meliloti (Beringer et al., 1987). In Gram Positive bacteria, chromosome transfer by plasmids has also been demonstrated (Stout and Iandolo, 1990).

Conjugal Transfer of *Agrobacterium* Ti Plasmid

DNA from *Agrobacterium tumefaciens* can be transferred to the host plant cell. The DNA known as T-DNA for transferred DNA, represents a defined segment on a large (200 kb) extrachromosomal element originally isolated from virulent cells of *Agrobacterium.* The plasmid was named pTi, for tumor-inducing plasmid (Zaenen et al., 1974). Most Ti plasmids failed to transfer when matings were conducted on normal laboratory media. However, the plasmid appeared to transfer at high frequencies when plant tumors induced by the virulent donor strain were inoculated with recipient cells (Kerr, 1969; Kerr 1971). Certain classes of opines produced by the crown gall tumor function as signals to induce conjugal transfer of the virulence plasmids. When donors harboring wild-type octopine-or-nopaline-type Ti plasmids are mated with recipients in the absence of the inducer opines, transconjugants rarely appear. Most of the rare transconjugants harbored spontaneous transfer-constitutive mutant derivatives of the Ti plasmid (Farrand, 1993).

The primary objective of this work was to investigate chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria*. The strategy of Pulsed-Field Gel Electrophoresis (PFGE) and a rare-cutting endonuclease in addition to growth on antibiotic media and plasmid profiles were undertaken to identify complete genome of donor, recipient and putative conjugants. Horizontal chromosomal gene transfer was determined by Southern hybridiza-

tion. The natural occurrence of horizontal transfer of the following chromosomal genes from two donor strains to a recipient strain of the bacterium by conjugation was determined in pepper leaves: Copper resistance (*cop*), hypersensitive reaction and pathogenicity (*hrp*) (Bonas et al., 1991), pigmentation (*pig*) and kanamycin resistance (Tn*5* sequences).

CHAPTER 2
DETERMINATION OF HORIZONTAL CHROMOSOMAL GENE TRANSFER AMONG STRAINS OF *Xanthomonas axonopodis* pv. *vesicatoria* BY CONJUGATION *IN VITRO*

Introduction

The transfer of nonhomologous DNA with new properties from bacterium to bacterium is probably characteristic of the evolution of bacteria, and may facilitate adaptation of bacteria to new niches (Lan and Reeves, 1996).

Strains of *Xanthomonas axonopodis* pv. *vesicatoria*, which cause the bacterial spot disease of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) have a great diversity in terms of restriction fragment length polymorphism (Cooksey and Graham, 1989). The bacterium has also great diversity in plasmid content (Canteros et al., 1995); most of the avirulence genes (Minsavage et al., 1990a) and genes for resistance to copper and streptomycin are located on plasmids (Minsavage et al., 1990b; Stall et al., 1986). The plasmids that contain copper resistance genes are self-transmissible (Stall et al., 1986). Although plasmids may be the major vehicles for the transfer of genes, there is no report of chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria*.

The objective of this work is to investigate the presence of chromosomal gene transfer among strains *X. axonopodis* pv. *vesicatoria in vitro*.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study and their sources are listed in Appendix. All strains of *X. axonopodis* pv. *vesicatoria* were grown on nutrient agar (NA) (Becton Dickinson, Cockeysville, MD). Nutrient broth cultures (NB) were grown 24 hr on a rotatory shaker (150 rpm) at 28 ^{0}C. Strains of *Escherichia coli* were cultivated on Luria-Bertani medium at 37 ^{0}C (Miller, 1972). Conjugation between strains was performed on nutrient-yeast-glycerol agar (NYGA). All strains were stored in sterile tap-water at room temperature or in 30% glycerol at -70 ^{0}C, or both. Antibiotics were used to maintain selection for resistance markers at the following final concentrations: Tetracycline, 10 μg/ml; rifamycin, 80 μg/ml, nalidixic acid, 50 μg/ml; kanamycin, 50 μg/ml and streptomycin, 50 μg/ml.

Obtaining Double-Antibiotic Mutants of Selected Strains of *X. axonopodis* pv. vesicatoria

A strain which is copper resistant and contains a single 15-kb plasmid was selected as a donor strain. It was suspected that copper resistance genes in this strain were located on the chromosome and could be used as a chromosomal

marker in the demonstration of the chromosomal gene transfer. The bacterium was grown in 4 ml of NB containing copper sulfate (200 µg/ml) for 24 hr on a rotatory shaker (150 rpm) at 28 ^{0}C. A 100 µl suspension of the bacterium (10^{9} cfu/ml) was plated on NA containing streptomycin (50 µg/ml) to isolate streptomycin resistant colonies. The streptomycin resistance marker was used to detect the changes in numbers of the donor during mating. One of the streptomycin and copper resistant colonies was selected as a donor strain in the demonstration of copper resistance gene transfer during conjugation. Another strain which was rifamycin resistant was grown in 4 ml NB containing rifamycin for 24 hr on rotatory shaker at 28 ^{0}C; 100 µl of the bacterial suspension (10^{9} cfu/ml) was plated on NA containing nalidixic acid to isolate a nalidixic acid and rifamycin resistant strain. One of the rifamycin and nalidixic acid resistant colonies was selected as a recipient strain for all conjugation tests. This was essential to eliminate the possibility of any rifamycin mutation of the donor strain during selection of conjugants. Another strain of *X. axonopodis* pv. *vesicatoria*, which was streptomycin resistant was selected as another donor strain. The Tn*5* (kanamycin resistance) transposon was randomly inserted into the chromosome of the bacterium essentially as described by Ruvkun and Ausubel (1981). The kanamycin resistance was used as a genetic marker in tests in the demonstration of the chromosomal gene transfer.

Test for *recA*

The recipient strain (82-8) of *X. axonopodis* pv. *vesicatoria* was tested for presence of *recA* gene by comparing growth of the known *recA*$^-$ strain (ED8767) and a *recA*$^+$ strain (RR1) of *E. coli*. The bacterial strains were streaked onto NA, and three-quarters of the streaks were exposed to UV light of a Edgegard hood for 10, 20, and 30 seconds. The remaining one quarter of each streak was not exposed to UV light as described by Maniatis et al. (1982). The exposed plate was incubated at 28 ^{0}C for 48 hr and growth of the bacteria was observed (Fig. 2-3).

Conjugation and Determination of Optimal Mating Time for Chromosomal Gene Transfer

Both donor and recipient strains were grown overnight at 28 ^{0}C on NA containing proper antibiotics. Bacteria were collected from solid media, suspended in 1 ml sterile tap water, washed two times by centrifugation, and resuspended in 50 µl sterile tap water. Donor (10^{10} cfu/ml, 50 µl) and recipient cells (10^{10} cfu /ml, 50 µl) were mixed very well by pipetting, spotted on NYGA medium, and incubated for 0, 8, 16, 24, 48, 72 and 96 hr at 28 ^{0}C. At the designated times, bacteria in the spots were resuspended in 800 µl sterile tap-water and vortexed strongly to stop the mating. All of the bacterial suspension (800 µl) was plated on two plates of media (400 µl for each plate) containing copper (200 µg/ml), rifamycin (80 µg/ml), and nalidixic acid (50 µg/ml) or kanamycin (50 µg/ml) rifamycin (80 µg/ml) and nalidixic acid (50 µg/ml) depending upon

donor strains used. Bacteria were incubated for 72 hr for detection of putative conjugants.

Plasmid Extraction

Bacterial strains were grown for 20 to 30 hr at 28 °C with vigorous shaking in tubes containing 3 ml of nutrient broth and proper antibiotics. Cells were harvested by centrifugation, cell density adjusted to 0.3 A_{600} in sterile deinozed-water, and plasmid DNA extracted by the method of Kado and Liu (1981). Plasmid DNA was subjected to electrophoresis until a bromophenol blue front had moved 10 cm from the well. The electrical current was 5V/cm for the submerged gel (Model H5, BRL). Samples were electrophoresed through 0.5% agarose gels (Sea Kem, DNA grade; FMC Corporation, Rockland, ME) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0), stained with ethidium bromide (0.5 µg/ml) for 30 min, and photographed using transmitted UV light. In each gel a plasmid preparation of *Erwinia stewartii* (Es) strain SW2 (Coplin et al., 1981) was included as a molecular weight marker.

The presence of any large plasmid in the donor, recipient and conjugants was determined by Pulsed-Field Gel Electrophoresis (PFGE) technique. Agarose blocks containing total intact genomic DNAs from donor, recipient and conjugants and other strains of *X. axonopodis* pv. *vesicatoria*, which have large plasmids, were run by PFGE and compared with a low-range marker resolvable in the range of 200 to 6.5 kb under the conditions described in the following section (Fig. 2-6).

Preparation of Genomic DNA in Agarose Blocks

Strains of *X. axonopodis* pv. *vesicatoria* were grown for 20 hr at 28 ^{0}C with vigorous shaking in tubes containing 3 ml of nutrient broth and proper antibiotics. The optical density at 600 nm of the suspension was adjusted to 0.3, and the cells were pelleted at 14,000 rpm for 2 min, washed in 1 ml of sterile deionized water, followed by centrifugation at 14,000 rpm for 2 min. After resuspension of the cells in 500 µl of TE buffer (10mM Tris-HCl pH:8.0, 1 mM EDTA pH: 8.0), the suspension was mixed with an equal volume of melted and cooled (55 ^{0}C) low-melting-point agarose (LMP) solution (1M Tris-HCl pH: 8.0, 1M $MgCl_2$, 250 mM EDTA pH: 8.0 and 2% (w/vol) LMP [FMC, Bio Products, Rockland, ME] in sterile deonized-water). This cell-agarose suspension was then pipetted into a mold chamber (Bio-Rad, Richmond, California, USA) and placed at 4 ^{0}C for 20 min to allow the blocks to solidify. The bacteria in the blocks were then placed in a lysing solution (250 mM EDTA pH: 9.5, 25% N-Lauroylsarcosine [Sigma Chemical, St, Louis, MO, sodium salt] and 1 mg/ml proteinase K [Sigma Chemical] in sterile deionized-water) for 24 hr in a waterbath at 50 ^{0}C. The agarose blocks were stored in 250 mM EDTA (pH: 8.0) at 4 ^{0}C.

Restriction Endonuclease Digestions

Agarose blocks were sectioned into 4 to 5 pieces about 3-4 mm section across the width of the block with a sterile glass coverslip. The small agarose pieces were rinsed two times in 1 ml of TE buffer for 1 h. The buffer was

changed, and blocks were transferred into Eppendorf tubes containing 100 µl of 1X restriction *Spe*I enzyme buffer. After 20 min, the buffer was replaced with fresh enzyme buffer, and 18 units of *Spe*I (Promega, Madison, WI) was added. The DNA was digested overnight at 37 °C.

Pulsed-Field Gel Electrophoresis

Fragments of the digested DNA were separated into fragments electrophoretically in 1% Sea-Kem-GTG agarose (FMC Bioproducts, Rockland, ME) in 0.5X TBE buffer (44.5 mM Tris-HCl, 44.5 mM Boric acid, 1mM EDTA pH 8.0) by using a Bio-Rad DRII Contour-Clamped Homogeneous Electric Field (CHEF) apparatus (Chu et al., 1986). The blocks containing the digested DNA were washed with a 500 µl wash solution (250 mM EDTA pH:9.5, 25% N-lauryl-sarcosine in sterile deinozed-water) at 55 °C for 2 hr and were rinsed one time in 500 µl wash solution for 15 min. The agarose blocks were then placed into 1-cm wide wells in a 14x12.5 cm agarose gel and sealed with 2% LMP agarose at 65 °C. The gel was placed in a Bio-Rad CHEF-DRII unit containing 1.6 L of 0.5X TBE buffer which was cooled to 14 °C throughout the run. The gels were run using a pulse time ramped from 5 to 45 s at 200V for 22 hr and a pulse time ramped from 1 to 13 s at 200V for 12 hr for separation of the size range 2200-225 kb and 0.13-194 kb, respectively.

Gels were stained with 0.5X TBE buffer containing ethidium bromide (0.5 µg/ml) for 30 min and destained with 0.5X TBE buffer for 30 min. After

electrophoresis, sizes of DNA fragments were determined by measuring distances of band migration compared with the yeast *Saccharomyces cerevisiae* chromosome marker (Bio-Rad Laboratories, Richmond, Calif.) and low range PFGE marker (New England-Biolabs).

Southern Hybridization Analysis

DNA separated in a PFGE gel was exposed to UV light for 2 min. DNA depurination was performed in 0.25 N HCl twice for 20 min on shaker at room temperature to enhance the large DNA fragment transfer. The gel was denaturated in 1.5 M NaCl-0.5 M NaOH and then neutralized in 0.5 M Tris-HCl-1.5 M NaCl pH 7.0 for 45 min on shaker at room temperature, respectively. The DNA was transferred to a nytran membrane (Schleicher & Schuell, Keene, NH) for 48 hr. The transfer buffer was 10X SSC (20X SSC is 3 M NaCl plus 0.3 M sodium citrate, pH: 7). After transfer, the membranes were baked for 1 h at 80 ^{0}C. Hybridization at high stringency was carried out at 68 ^{0}C in a standard hybridization buffer containing 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 1% blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN). The membranes were washed twice for 5 min in 2X SSC-0.1% SDS at room temperature and then twice for 15 min in 0.5X SSC-0.1% SDS at 65 ^{0}C after hybridization. The hybridizations were detected by Genius non-radioactive Chemiluminescent DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Post-hybridization washes and chemiluminescent development were carried out according to the instructions of the manifacturer. For reuse, these membranes were washed in distilled water for 1

min and then the probe was removed by soaking them two times in 0.4 N NaOH plus 0.1% (w/v) SDS for 20 min at 37 ^{0}C on shaker. Finally, the membranes were rinsed in 2X SSC for 2 min and membrane was dried at room temperature until using.

Results

Growth of Donor, Recipient and Conjugants on Antibiotic Media

Donor Cu^{r} Str^{r}, recipient Rif^{r} Nal^{r} and putative conjugants Cu^{r} Rif^{r} Nal^{r} were tested for growth on antibiotic media. The donor strain was able to grow on media containing copper and streptomycin, but not on media containing rifamycin and nalidixic acid, or containing copper, rifamycin and nalidixic acid (Fig. 2-1). The recipient strain grew on media containing rifamycin and nalidixic acid, but not copper and streptomycin, or copper, rifamycin and nalidixic acid (Fig. 2-1). It was determined that putative conjugants grew on media containing rifamycin and nalidixic acid, and copper, but most did not grow on media containing copper and streptomycin. A few putative conjugants grew on media containing copper and streptomycin (Fig. 2-1).

Similarly, donor Kn^{r} Str^{r}, recipient Rif^{r} Nal^{r} and conjugants Kn^{r} Rif^{r} Nal^{r} were tested for growth on antibiotic media. The donor strain grew on media containing kanamycin and streptomycin, but not on media containing rifamycin and nalidixic acid, and kanamycin, rifamycin and nalidixic acid (Fig. 2-2). The recipient strain grew on media containing rifamycin and nalidixic acid, but not on kanamycin and streptomycin, and kanamycin, rifamycin and nalidixic acid

Fig. 2-1. Growth of donor Cu^r Str^r, recipient Rif^s Nal^r and conjugant Cu^r Rif^s Nal^r on antibiotics media. A, B, C: Growth of donor, recipient and conjugant on medium containing copper and streptomycin, respectively. D, E, F: Growth of donor, recipient and conjugant on medium containing rifamycin and nalidixic acid, respectively. G, H, I: Growth of donor, conjugant and recipient on medium containing copper, rifamycin and nalidixic acid, respectively.

Fig. 2-2. Growth of donor $Kn^r Str^r$, recipient $Rif^s Nal^r$ and conjugant $Kn^r Rif^s Nal^r$ on antibiotic media. A, B, C: Growth of donor, recipient and conjugant on medium containing kanamycin and streptomycin, respectively. D, E, F: Growth of donor, recipient and conjugant on medium containing rifamycin and nalidixic acid, respectively. G, H, I: Growth of donor, recipient and conjugant on medium containing kanamycin, rifamycin and nalidixic acid, respectively.

(Fig. 2-2). The putative conjugants grew on media containing rifamycin and nalidixic acid, and kanamycin, rifamycin and nalidixic acid, but not on kanamycin and streptomycin (Fig. 2-2).

Evidence for Presence of *rec*A Gene for Recipient Strain of *X. axonopodis* pv. *vesicatoria*

The recipient strain 82-8 $Rif^{s}Nal^{r}$ of *X. axonopodis* pv. *vesicatoria* grew on NA medium after different exposure time to UV. Even 30 seconds exposure of UV did not affect growth of the bacterium (Fig. 2-3). Similarly, all exposure times to UV did not affect growth of strain RR1 *rec*A^{+} strain of *E. coli*. On the other hand, even 10 seconds exposure of UV stopped the growth of ED8767 *rec*A^{-} strain of *E. coli* (Fig. 2-3).

Plasmid Profiles of Donor, Recipient and Conjugants

The donor $Cu^{r}Str^{r}$ strain had a 15 kb plasmid, and recipient $Rif^{s}Nal^{r}$ strain has three plasmids in size of about 120 kb, 44 kb and 33 kb. Putative conjugants growing on $Cu^{r}Rif^{s}Nal^{r}$ had exactly the same plasmid profile as recipient. A plasmid 15 kb in size was not detected in the plasmid profile of the conjugants (Fig. 2-4).

The donor $Kn^{r}Str^{r}$ strain had two plasmids in size of about 300 kb and 40 kb. The recipient $Rif^{s}Nal^{r}$ strain had three plasmids in size of about 120 kb, 44 kb and 33 kb. Putative conjugants $Kn^{r}Rif^{s}Nal^{r}$ had exactly the same plasmid profile with recipient in size of about 120 kb, 44 kb and 33 kb. Any plasmid in

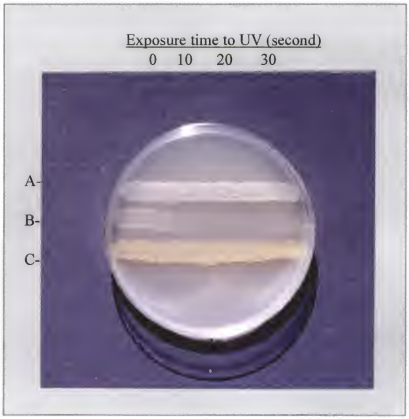

Fig. 2-3. Determination of presence of *rec*A gene in recipient Rif^s Nal^r strain of *Xanthomonas axonopodis* pv. *vesicatoria*. A, RR1 *rec*A^+ strain of *Escherichia coli*; B, ED8767 *rec*A^- strain of *E. coli*; C, recipient (82-8) strain of *X. axonopodis* pv. *vesicatoria*.

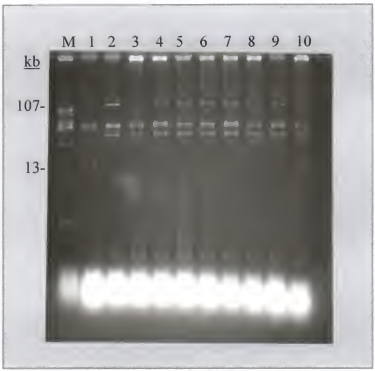

Fig. 2-4. Agarose gel electrophoresis of plasmid DNA from donor, recipient and conjugants. Lanes: M, plasmids of *Erwinia stewartii*; 1, donor Cu^r Str^r; 2, recipient Rif^s Nal^r; 3-10, conjugants Cu^r Rif^s Nal^r.

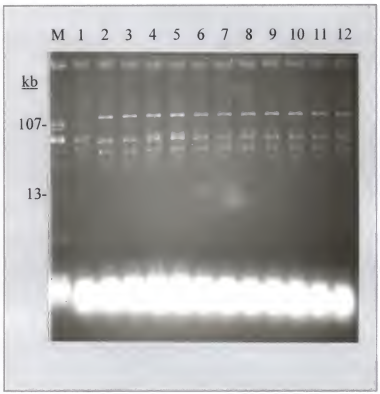

Fig. 2-5. Agarose gel electrophoresis of plasmid DNA from donor, recipient and conjugants. Lanes: M, plasmids of *Erwinia stewartii*; 1, donor Kn^r Str^r; 2, recipient Rif^s Nal^r; 3-12, conjugants Kn^r Rif^s Nal^r.

size of about 300 kb and 40 kb was not detected in plasmid profile of the conjugants (Fig. 2-5).

The presence of a large plasmid (<300 kb) was not detected in either donor, recipient, or putative conjugant in PFGE tests. A large plasmid was detected in strains, E3 and 75-3 of *X. axonopodis* pv. *vesicatoria* (Fig. 2-6).

Total Genome Profiles of Donor, Recipient, and Conjugants

The total genome profiles of donor, recipient and putative conjugants from different mating times were determined by PFGE and a rare-cutting enzyme, *Spe*I. Putative conjugants obtained from mating of donor Cu^r Str^r and Rif^s Nal^r mainly shared a genome profile with the recipient strain except for some polymorphism, but conjugants were not similar to donor strain. In all matings with different incubation times, genome profiles of conjugants were stable except for some minor differences (Fig. 2-7 through 2-14).

Similarly, putative conjugants obtained after mating between donor Kn^r Str^r and Rif^s Nal^r mainly shared similar genome profile with the recipient except for some polymorphisms, but were not similar to donor strain (Fig. 2-16, 2-17).

Transfer of Chromosomal Copper Genes and Tn*5* Sequences from Donor to Recipient

Horizontal transfer of chromosomal copper genes and Tn*5* sequences were demonstrated by probing the gel after PFGE and Southern hybridization with DNA from clones carrying copper genes and Tn*5* sequences. Copper genes were

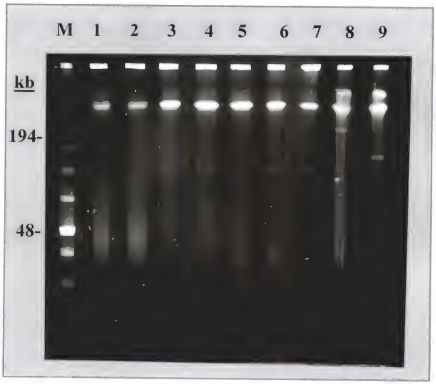

Fig. 2-6. PFGE of undigested total genomic DNA of donor $Cu^r Str^r$, recipient $Rif^r Nal^r$, conjugants $Cu^r Rif^r Nal^r$, E3 and 75-3 strains of *X. axonopodis* pv. *vesicatoria*.Lanes: M, low range marker; 1-2, the donor; 3-4, the recipient; 5-7, the conjugants; 8, strain 75-3; 9, strain E3.

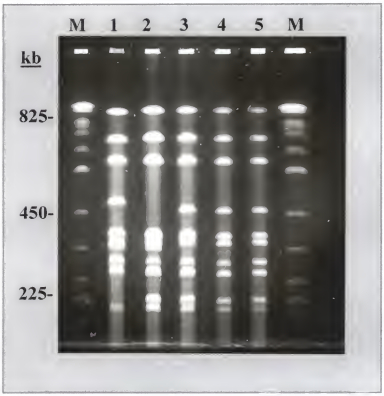

Fig. 2-7. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 8 hr-mating. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-5, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

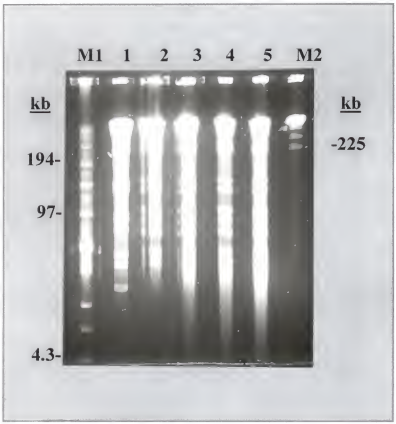

Fig. 2-8. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 8 hr-mating. Lanes: M1, Low-range PFGE marker; M2, DNA size standards of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-5, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

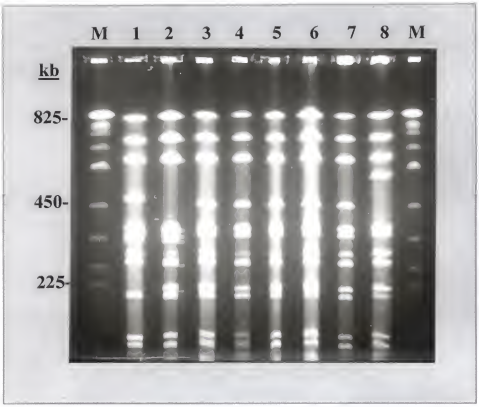

Fig. 2-9. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 24 hr-mating. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

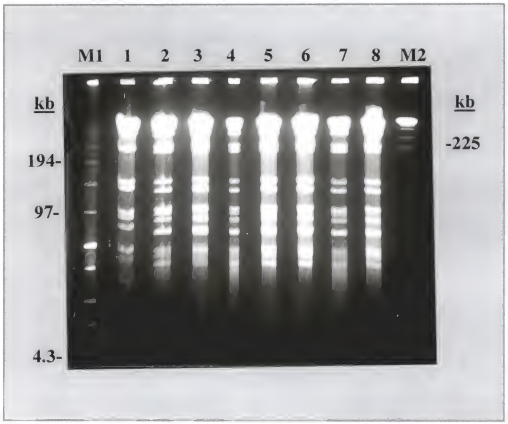

Fig. 2-10. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 24 hr-mating. Lanes: M1, Low-range PFGE marker; M2, DNA size standards of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

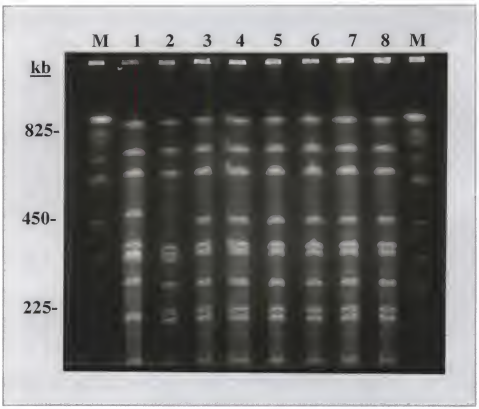

Fig. 2-11. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 48 hr-mating. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

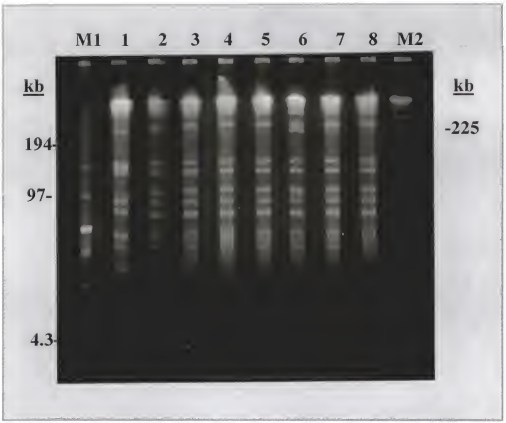

Fig. 2-12. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 48 hr-mating. Lanes: M1, Low-range PFGE marker; M2, DNA size standards of *Sacchoromyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

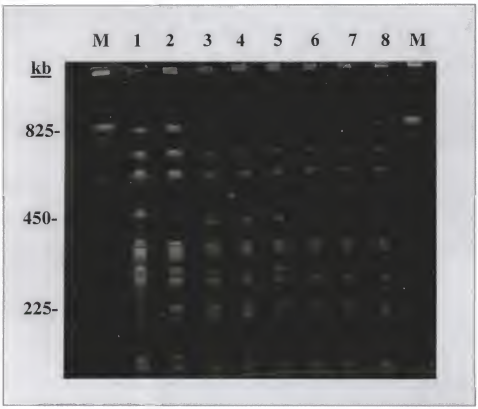

Fig. 2-13. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 72 hr-mating. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

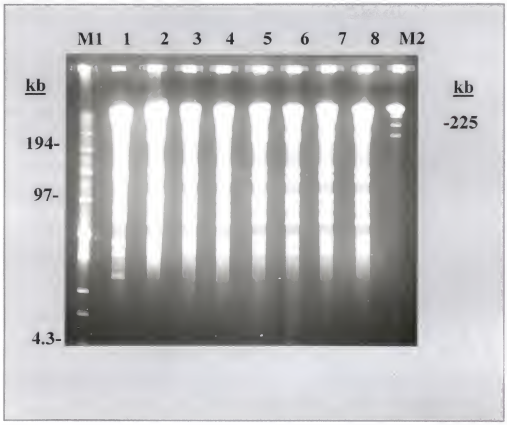

Fig. 2-14. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 72 hr-mating. Lanes: M1, Low-range PFGE marker; M2, DNA size standardts of *Saccharomyces cerevisiae*; 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to copper, rifamycin and nalidixic acid. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

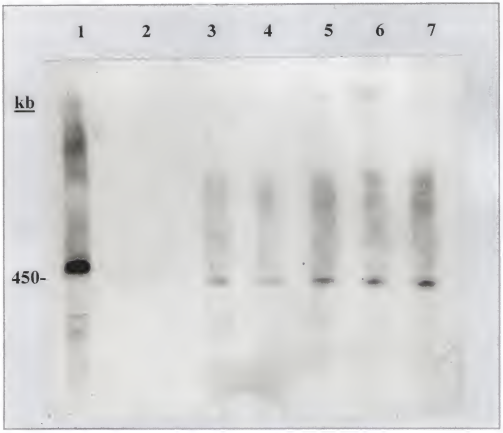

Fig. 2-15. Hybridization of copper gene cluster of *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: 1, donor resistant to copper and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-7, conjugants resistant to copper, rifamycin and nalidixic acid. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel.The ramped pulse times were 5-45 s for 22 hr at 14°C and 200 volt. The clone containing copper resistance genes was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

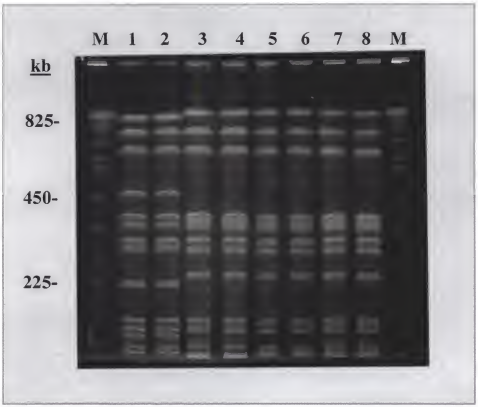

Fig. 2-16. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 72 hr-mating. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1-2, donor resistant to kanamycin and streptomycin; 3-4, recipient resistant to rifamycin and nalidixic acid; 5-8, conjugants resistant to kanamycin, rifamycin and nalidixic acid. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

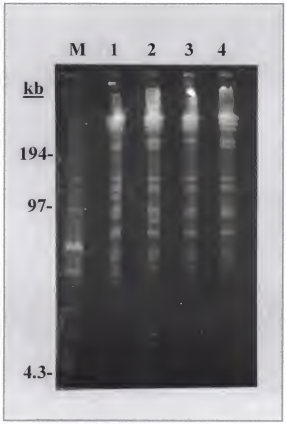

Fig. 2-17. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from 72 hr-mating. Lanes: M, Low-range PFGE marker; 1, donor resistant to kanamycin and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-4, conjugants resistant to kanamycin, rifamycin and nalidixic acid. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 volt. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer.

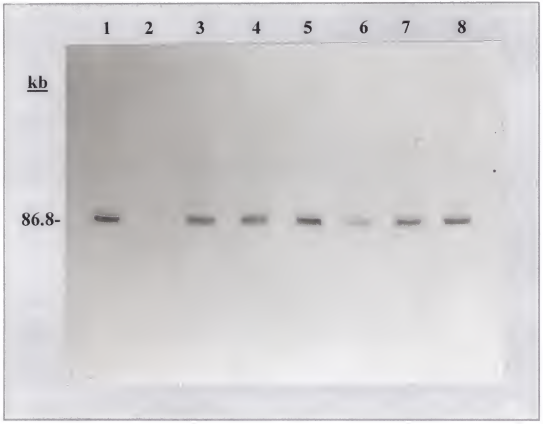

Fig. 2-18. Hybridization of a clone containing Tn*5* sequences to genomic DNA of donor and conjugants of *X. axonopodis* pv. *vesicatoria*. Lanes: 1, donor resistant to kanamycin and streptomycin; 2, recipient resistant to rifamycin and nalidixic acid; 3-8, conjugants resistant to kanamycin, rifamycin and nalidixic acid. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 volt. Tn*5* sequences (2.96 kb) was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

located on a nearly 500 kb DNA fragment of donor strain restricted by *Spe*I and separated by PFGE. They were detected on a nearly 450 kb DNA fragment from the putative conjugants restricted by *Spe*I. There was not any hybridization of copper genes with DNA of recipient strain (Fig. 2-15). The Tn*5* sequences inserted on the chromosome of donor Kn^r Str^r strain were detected on both donor and putative conjugants by using the probe of Tn*5* sequences on digested total genomic DNA restricted by *Hind*III and *Eco*RI and separated by electrophoresis. Tn*5* sequences were not detected on the recipient strain (Fig. 2-18).

Determination of Optimal Mating Time for Conjugation *in vitro*

The frequency of conjugation *in vitro* increased with increasing mating time from 0 h to 8, 16, 24, 48, and 72 hr (Table 2-1). Conjugants were not detected in zero time mating (without incubating bacterial mixture on NYGA medium). After 8 hr mating only one conjugant was obtained *in vitro*. The frequency of the appearence of Cu^r Nal^r Rif^r resistant colonies decreased after 72 hr mating time (Table 2-1). However, nearly 200 conjugants were obtained after 72 hr mating.

Discussion

Mating for conjugations is usually made with strains that have double markers in each strain. The double markers are used to prevent selection of false conjugants that might arise from mutation of one of the markers. In this work

Table. 2-1. Optimal mating-time for transfer of chromosomal copper genes from donor to recipient strain of *X. axonopodis* pv. *vesicatoria in vitro*.

Mating-time (hour)	Frequency of conjugation (per donor)
0	0
8	$1x10^{-9}$
16	$6.6x10^{-9}$
24	$1.53x10^{-8}$
48	$1.56x10^{-8}$
72	$6.43x10^{-8}$
96	$5.53x10^{-8}$
120	$2.1x10^{-8}$

double markers were present in each donor and each recipient, but in selection of putative conjugants in this work only one marker was present in the donor and two were present in the recipient (Fig. 2-1, 2-2). When two markers were used in the donor for selection of conjugants, no conjugants appeared. Evidently no conjugants appeared because the two markers in the donors (copper, or kanamycin resistance) were not linked closely with the streptomycin resistance locus. Thus, copper and streptomycin resistance, or kanamycin and streptomycin resistance were too far from each other for recombination of both markers to occur in the recipient.

A single marker, either copper resistance or kanamycin resistance, was then used as a marker for chromosomal movement in conjugations. No mutants for these resistances in the recipient strain were ever seen on media containing those antimicrobial agents. Involvement of at least four structural genes needed for copper resistance probably precluded the development of copper resistance by mutation. It is not known at this time why mutations for kanamycin resistance are very low in the recipient strain of *X. axonopodis* pv. *vesicatoria*. Nevertheless, the low number of putative conjugants obtained after matings on artifical media might have been the result of rare mutations for copper resistance and kanamycin resistance in the recipient strain. Therefore, it was necessary to confirm that chromosomal transfer occurred by other procedures. First, it was necessary to determine that the conjugants were similar to the recipient in DNA background. This was done in two ways. In the first way, plasmid profiles of the recipient and the donors were different. In the second way, the total genomic

DNA profiles after restriction with a rare-cutting endonuclease were determined. Different profiles were seen for the donors and the recipient, but were more nearly like the recipient. Some polymorphism occurred between the putative conjugants and the recipients in comparisons. After it was determined that the conjugants were like the recipient, the putative conjugants were probed with the genes for copper resistance that existed in one donor and with the genes for kanamycin resistance which existed on the transposon Tn*5* that was inserted in a second donor strain. The detection of copper resistance genes and Tn*5* sequences being present in the putative conjugants, along with the plasmid profiles and DNA fragment profiles that were similar to the recipient were evidenced that the putative conjugants were in fact true conjugants.

Copper resistance genes are located mostly on 188-200 kb self-transmissible large plasmids (Bender et al., 1990; Stall et al., 1986) and on 100 kb non-mobilizable plasmid (Cooksey et al., 1990) in *X. axonopodis* pv.*vesicatoria*. The donor strain did not have such a large plasmid (Fig. 2-4), but a large plasmid undetected by the Kado and Liu technique (Kado and Liu, 1981) might have been present. Therefore, we checked for a megaplasmid by PFGE and did not find one (Fig. 2-6). Thus, the copper resistance in XvP26 was not plasmid-borne. The copper genes were subsequently located on a 450 kb fragment of chromosomal DNA of the bacterium by a Southern hybridization test.

Major evidence for chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria* in this work was obtained by examining total genomic DNA fragment profiles of donor, recipient and conjugants by PFGE, after

digestion with the rare-cutting endonuclease, *Spe*I. Using two different running programs, PFGE provided an excellent opportunity to resolve large and small fragments of the total genomic DNA restricted by *Spe*I. Sizes of fragments of chromosomal DNA of the recipient were changed by newly integrated DNA from the donor strain. In the case of the conjugants Kn^r Rif^s Nal^r the exact same fragment profile as the recipient occurred when DNA fragments in the range of 2200-225 kb were restricted with *Spe*I and separated with PFGE using 5-45 s pulse time in running program (Fig. 2-16). However, some polymorphism was detected in fragment profiles between 48-97 kb separated with PFGE using 1-13 s in running program that resolves DNA fragment in the range of 6.5-200 kb (Fig. 2-17). Therefore, recombination of new non-homologous DNA containing the Tn*5* locus from the donor strain probably occurred in this region (48-97 kb) of the recipient chromosome. This was confirmed by Southern hybridization by using Tn*5* sequences as a probe. Hybridization was detected with a DNA fragment 86.8 kb in size (Fig. 2-18).

The conjugants Cu^r Rif^s Nal^r had a unique 450-kb DNA fragment (Fig. 2-7, 2-9, 2-11, 2-13). The copper gene cluster hybridized to the fragment in Southern hybridization (Fig. 2-15). Therefore, it is likely that recombination of the donor fragment occurred in this region (450-kb) of the recipient chromosome. Polymorphism was not detected in the range of 6.5-200 kb DNA fragment in these conjugants (Fig. 2-8, 2-10, 2-12, 2-14). Separation of restricted fragments of the donors, recipient and conjugants by PFGE and localization of transferred

chromosomal genes by Southern hybridization has given a conclusive and clear demonstration of chromosomal gene transfer under these experimental conditions.

In the experiments to determine the optimal mating time for chromosomal copper genes and kanamycin resistance, conjugation frequency increased from starting 0 to 72 hr and then decreased (Table 2-1). The question, "does this increase occur because of multiplication of conjugants obtained in 8 hr, or does transfer of chromosomal genes occur throughout the 72 hr?" To find an answer to this question, I determined the cell numbers of conjugants, donor and recipient over 3 days of mating. There was no significant increase of the numbers of donor and recipient , but conjugants increased (data not included). The donors and recipients may not increase because of a very high concentration of donor and recipient cells on the media. A decrease in conjugation frequency with increasing mating time after 3 days may be related to the survival of the bacterium on the media. In conclusion, most conjugants were obtained after 3 days of mating under these experimental conditions *in vitro*.

CHAPTER 3
CLONING OF CHROMOSOMAL COPPER RESISTANCE GENE CLUSTER FROM *Xanthomonas axonopodis* pv. *vesicatoria*

Introduction

Copper compounds have been used for decades for the control of bacterial and fungal plant pathogens, but copper-resistant bacterial pathogens have recently been reported (Adaskaveg and Hine, 1985; Bender and Cooksey, 1986; Marco and Stall, 1983; Sundin et al., 1989; Lee et al., 1994). Most of the genes for copper resistance from plant pathogenic bacteria (Bender and Cooksey, 1986; Bender et al., 1990; Cooksey, 1987; Cooksey, 1990a; Cooksey, 1990b; Stall et al., 1986; Voloudakis et al., 1993) and some other bacteria including *Escherichia coli* (Tetaz and Luke, 1983) and *Mycobacterium scrofulaceum* (Erardi et al., 1987) are plasmid encoded. Chromosomal genes for copper resistance have been cloned from *X. arboricola* pv. *juglandis* (Lee et al., 1994).

Copper resistance genes in *X. axonopodis* pv. *vesicatoria* from Florida and Oklahoma were located on 188-200 kb self-transmissible plasmids (Bender et al., 1990; Stall et al., 1986), and also are located on a non-self-transmissible plasmid 100 kb in size (Cooksey et al., 1990). Although copper resistance genes from *Xanthomonas spp.* have the same general *cop*ABCD structure as *Pseudomonas syringae*, there is some difference in gene size and DNA sequences. There is also no report on the presence of the regulatory genes, *cop*RS,

in *X. campestris* (Lee et al., 1994). Copper resistance genes were used as a genetic marker for chromosomal movement in conjugations because copper resistance is encoded by multiple genes that minimized mutation for copper resistance of a recipient strain. Cloned copper resistance genes from other plant pathogenic bacteria were examined for hybridization with copper genes from the donor, but total genomic DNA of the donor did not hybridize with these other resistance gene copper genes. Therefore, it was essential to clone copper genes from the donor strain for use as a probe for the genes in putative conjugants.

The objective of this work is to clone the copper gene cluster from strain XvP26 of *X. axonopodis* pv. *vesicatoria*, determine its genomic location and to compare with other copper resistance genes from plant pathogenic bacteria.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study and their sources are listed in Appendix. A copper-sensitive strain (82-8) of *X. axonopodis* pv. *vesicatoria* was grown on nutrient agar (Becton Dickinson, Cockeysville, MD). Nutrient broth (NB) cultures were grown 24 hr on a rotatory shaker (150 rpm) at 28 ^{0}C. Strains of *Escherichia coli* were cultivated on Luria-Bertani (LB) medium at 37 ^{0}C (Miller, 1972). A cosmid, pLAFR3, from a library of donor strain (XvP26) of *X. axonopodis* pv. *vesicatoria* was provided by J. Minsavage, University of Florida, and maintained on LB media containing tetracycline at 4 ^{0}C. Triparental matings were performed on nutrient- yeast-glycerol agar (NYGA).

All strains were stored in sterile tap-water at room temperature or in 30% glycerol at -70 ^{0}C, or both. Antimicrobial agents were added to media to maintain selection for resistance markers at the following final concentrations: Tetracyline, 10 μg/ml; rifamycin, 80 μg/ml; copper sulfate, 200 μg/ml.

General DNA Manipulations

Miniscale preparations of *E. coli* plasmid DNA were made by an alkaline lysis method as describe by Sambrook et al. (1989). Cloned DNA fragments containing copper resistance genes from different plant pathogenic bacteria were isolated for Southern blot hybridization by digesting with appropriate restriction enzymes according to conditions specified by the manufacturer. The restricted DNAs were separated by electrophoresis in 0.7% agarose gel (Seakem GTG, FMC Bioproduct, Rockland, ME) in TAE buffer at 5V/cm. The gel was stained with ethidium bromide (0.5 μg/ml) for 30 min and then photographed over a UV transluminator with type 55 polaroid film. The gel was used for Southern hybridization to compare the copper gene cluster from strain XvP26 with copper resistance genes from different plant pathogenic bacteria,

Bacterial Conjugation and Isolation of Copper-Resistance Clone

Triparental matings (Figurski and Helinski, 1979) were carried out by mixing mid-log growth phase cells of 82-8 Rif^{r} of *X. axonopodis* pv. *vesicatoria* as the recipient with *E coli* DH5α (cosmid clones) as the donor and HB101 (PRK2073) as the conjugational helper. The volume ratio of recipient-donor-helper was 2:1:1. The mixture was spread onto NYGA (Nutrient yeast glycerol

rifamycin, tetracycline and copper sulfate (20 μg/ml). Transconjugant colonies were plated onto NA media amended with 200 μg/ml of copper sulfate to detect clones carrying copper resistance genes from pLAFR3 cosmid library of *X. axonopodis* pv. *vesicatoria* XvP26.

The clone carrying the copper-resistance gene cluster was subcloned by digesting insert DNA with appropriate restriction endonucleases and ligating fragments into pLAFR3 vector. Ligations were performed with T4 DNA ligase, according to manufacturers' instructions. Each fragment obtained by digestion of *Eco*RI and *Hind*III and separated by electrophoresis was purified from agarose gel by the Wizard PCR Preps DNA purification system (Promega, Madison,WI). The vectors with insertions were transformed into *E. coli* DH5α. Competent cells of *E. coli* were produced by the calcium chloride procedure as described by Sambrook et al. (1989).

Pulsed Field Gel Electrophoresis

The strain of *X. axonopodis* pv. *vesicatoria* XvP26 was grown for 20 hr in tubes containing 3 ml nutrient broth with proper antibiotics at 28 ^{0}C with vigorous shaking. The optical density at 600 nm of the suspension was adjusted to 0.3, and the cells were pelleted at 14,000 rpm for 2 min, washed in 1 ml of sterile deionized-water, followed by centrifugation at 14,000 rpm for 2 min. After resuspension of the cells in 500 μl of TE buffer (10 mM Tris-HCl pH:8.0, 1 mM EDTA pH: 8.0), the suspension was mixed with an equal volume of melted and cooled (at 55 ^{0}C) low-melting-point agarose (LMP) solution (1M Tris-HCl pH:8.0; 1 M $MgCl_2$, 250 mM EDTA pH:8.0, 2% (w/vol) LMP agarose [FMC, Bio, Product, Rockland, ME] in sterile deionized-water) and this cell

suspension agarose mixture was pipetted into a mold chamber (Bio-Rad, Richmond, California, USA) and placed at 4 ^{0}C for 20 min to allow the blocks to solidify. The agarose blocks were placed in lysing solution (250 mM EDTA pH: 9.5, 25% N-Lauroylsarcosine (Sigma Chemical, St. Louis, MO, sodium salt) and proteinase K (1 mg/ml) (Sigma Chemical) in sterile deionized-water) for 24 hr at 50 ^{0}C in waterbath. The agarose blocks were stored in 250 mM EDTA (pH: 8.0) at 4 ^{0}C.

The agarose block were sectioned into 4 to 5 pieces about 3-4 mm section across the width of the block with a sterile glass coverslip. The small agarose pieces were rinsed two times in 1 ml of TE buffer for 1 h. The buffer was changed, and blocks were transferred into containing 100 µl of 1X restriction enzyme buffer of *Spe*I. After 20 min, the buffer replaced with fresh enzyme buffer, and 18 unit of *Spe*I (Promega, Madison, WI) was added. The DNA was digested overnight at 37 ^{0}C.

The blocks were washed with a 500 µl wash solution for 15 min before loading onto agarose gel. The agarose blocks were placed into wells of the gel and sealed with 2% LMP agarose at 65 ^{0}C. DNA digests were separated electrophoretically using a Bio-Rad DRII Contour-Clamped Homogeneous Electric Field (CHEF) apparatus (Chu et al., 1986). 1% Sea-Kem-GTG agarose (FMC Bioproducts, Rockland, ME) in 0.5X TBE buffer (44.5 mM Tris-HCl, 44.5 mM Boric acid, 1mM EDTA pH:8.0) was placed in a Bio-Rad CHEF-DRII unit containing 1.6 L of 0.5X TBE buffer which was cooled to 14 ^{0}C throughout the run. The gels were run using a pulse times ramped from 5 to 45 s at 200V for 22 hr and pulse times ramped from 1 to 13 s at 200V for 12 hr. These times and voltage conditions were used for separation of the size range 2200-225 kb and

225-0.13 kb, respectively.

Gels were stained with 0.5X TBE buffer containing ethidium bromide (0.5 μg/ml) for 30 min and destained with 0.5X TBE buffer for 30 min. After electrophoresis, sizes of DNA fragments were determined by measuring distances of band migration compared with the yeast *Saccharomyces cerevisiae* chromosome marker (Bio-Rad Laboratories, Richmond, Calif.) and low-range PFGE marker (New England-Biolabs).

Southern Hybridization of DNA from Gels Generated by PFGE

DNA separated using both electrophoresis procedures was exposed to UV light for 2 min. DNA depurination was performed in 0.25N HCl for 20 min on a shaker at room temperature, to enhance large DNA fragment transfer. The gel was denatured in 1.5 M NaCl-0.5 M NaOH and then neutralized in 0.5 M Tris-HCl,1.5 M NaCl pH 7.0 for 45 min on shaker at room temperature, respectively. DNA was transferred to a nytran membrane (Schleicher & Schuell, Keene, NH) for 48 hr. The transfer buffer was 10X SSC (20X SSC is 3 M NaCl plus 3 M sodium citrate, pH:7). The membrane was baked for 1 h at 80 ^{0}C. Hybridization was carried out at 68 ^{0}C in a buffer containing 5X SSC, 0.1% N-lauroyl sarcosine 0.02% SDS, 1% blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN). Posthybridization washes at high-stringency were carried out at room temperature twice for 5 min in a buffer containing in 2X SSC-0.1% SDS and at 65 ^{0}C twice for 15 min in a buffer containing 0.5X SSC-0.1% SDS. Posthybridization washes in low-stringency were carried out at room temperature in a buffer containing 0.5X SSC. The hybridization were detected by Genius non-radioactive Chemiluminescent DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals Indianopolis, IN).

Results

Cloning and Subcloning of Copper resistance Genes from *X. axonopodis* pv. *vesicatoria* XvP26

A cosmid (pLAFR3) library of *X. axonopodis* pv. *vesicatoria* XvP26 was screened to detect clone(s) carrying copper gene cluster. Eleven hundred clones of a cosmid library were transferred into recipient strain (82-8) by triparental mating. Two cosmid clones conferred copper resistance to the 82-8 strain of *X. axonopodis* pv. *vesicatoria* (Fig. 3-1). A restriction enzyme map of one clone, which contained approximately 27.9 kb of insert DNA, was generated (Fig. 3-5). To further localize the copper gene cluster, different fragments of the insert were subcloned into pLAFR3 (Fig. 3-2). The subclones were transformed into *E.coli* DH5α, mobilized into 82-8 Cu^s and screened for copper resistance. One subclone which contained a 10.9-kb *Eco*RI-*Hind*III fragment conferred copper resistance to 82-8 on media containing 200 μg/ml of copper sulfate (Fig. 3-3, 3-4).

Localization of The Copper Genes on The Chromosome of *X. axonopodis* pv. vesicatoria

Copper genes were localized on the chromosome of the *X. axonopodis* pv. *vesicatoria* by Southern hybridization (Fig. 3-6B). The cloned copper gene was used as a probe. The genes were localized on approximately a 500-kb DNA fragment separated by PFGE and rare-cutting enzyme, *Spe*I, using 5-45 s running program (Fig. 3-6A).

There was no hybridization signal on fragments in the range of 200-6.5 kb DNA fragment separated by PFGE using 1-13 s running program.

Comparison of The Copper Resistance Genes with Other Copper Resistance genes from plant pathogenic bacteria

Cloned copper genes from *X. axonopodis* pv. *vesicatoria* XV26 were compared with plasmid-borne copper genes from *X. axonopodis* pv. *vesicatoria* 75-3 and *Pseudomonas syringae* pv. *tomato* and chromosomal-borne copper genes from *X. arboricola* pv. *juglandis* by Southern hybridization (Fig. 3-7A, 3-7B). The clone of copper genes from *X. axonopodis* pv. *vesicatoria* XvP26 was used as a probe. Weak hybridization signals were detected on the plasmid-borne and chromosomal-borne copper genes in low stringency conditions, but the probe showed very strong hybridization with itself (Fig. 3-7B).

Discussion

A unique chromosomal copper gene cluster was cloned from *X. axonopodis* pv. *vesicatoria* XvP26. The copper clone used as a probe hybridized weakly to plasmid-borne copper genes from *X. axonopodis* pv. *vesicatoria* 75-3 and *Pseudomonas syringae* pv. *tomato* and to chromosomal-borne copper genes from *X. arboricola* pv. *juglandis* in low stringency conditions (Fig. 3-7B). Copper genes from *X. arboricola* pv. *juglandis*, *X. axonopodis* pv.*vesicatoria* 75-3 and *P. syringae* pv. *tomato* did not hybridize to total DNA from *X. axonopodis* pv. *vesicatoria* XvP26 in high stringency conditions. In addition, the copper resistance gene cluster differed from other clones in restriction fragment lenght

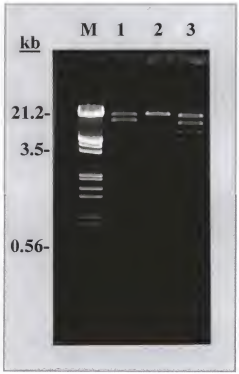

Fig. 3-1. Restriction endonuclease fragments of the cosmid clone carrying copper resistance gene cluster from *X. axonopodis* pv. *vesicatoria* restricted with *Eco*RI and *Hind*III. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III; 1, 2, 3, cosmid clone carrying copper resistance genes digested with *Eco*RI, *Hind*III and *Eco*RI-*Hind*III, respectively.

Fig. 3-2. Different subclones of the cosmid clone pLAFR3, which contains the copper resistance genes of *X. axonopodis* pv. *vesicatoria* XvP26. Lanes: M, phage λ, restricted with *Eco*RI and *Hind*III; 1, 2, 5, 6, 7, 9, 10, subclones carrying copper resistance genes and first fragment of the cosmid clone restricted with *Eco*RI and *Hind*III; 3, subclone of the third fragment of the cosmid clone, 4,8,11, subclones of the second fragment of the cosmid clone; 12, cosmid clone of pLAFR3 carrying copper resistance gene cluster restricted with *Eco*RI and *Hind*III.

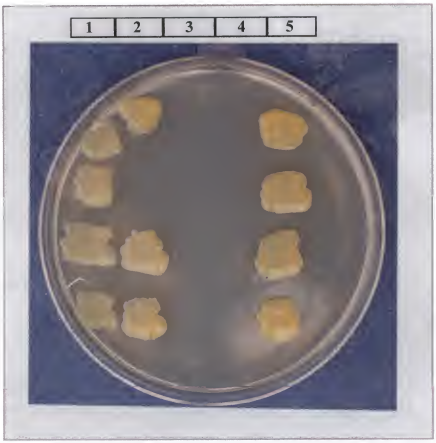

Fig. 3-3. Growth of *X. axonopodis* pv. *vesicatoria* 82-8 (Cu^s) on media containing 200 μg/ml copper sulfate after transferring subclones into it by triparental mating. 1, 2, 5, conferring copper resistance to 82-8 Cu^s by transferring the subclones: 1, 2, 5, (Fig. 3-2); 3, 4, no growth of the 82-8 Cu^s after transferring the subclones: 3, 4, (Fig. 3-2).

Fig. 3-4. Growth of *X. axonopodis* pv. *vesicatoria* 82-8 (Cu^s) on media containing 200 μg/ml copper sulfate after transferring subclones into it by triparental mating. 1, 2, 4, 5, conferring copper resistance to 82-8 Cu^s by transferring the subclones: 6, 7, 9, 10 (Fig. 3-2); 3, no growth of the 82-8 Cu^s after transferring the subclones: 8, (Fig. 3-2); 6, conferring of copper resistance to 82-8 Cu^s by transferring the cosmid clone of *X. axonopodis* pv. *vesicatoria* (Fig. 3-2).

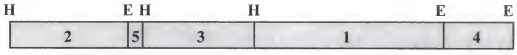

Fig. 3-5. Restriction endonuclease maps of the 27.9-kb DNA fragment carrying copper resistance genes of *X. axonopodis* pv. *vesicatoria* XvP26 restricted with *Eco*RI and *Hind*III. E, *Eco*RI; H, *Hind*III; 1, 10.9-kb fragment carrying the copper resistance gene cluster; 2, 3, 4, 5: 7.2, 5.3, 3.7, 0.7-kb second, third, fourth and fifth fragments, respectively.

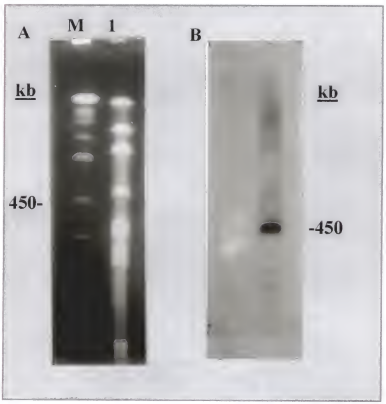

Fig. 3-6. (A) PFGE of *Spe*I digests of genomic DNA of *X. axonopodis* pv. *vesicatoria* XvP26. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, *X. axonopodis* pv. *vesicatoria* XvP26 resistant to copper. The ramped pulse times were 5-45 s for 22 hr at 14 °C and 200V. The gel was 1% Seakem-GTG agarose in 0.5X TBE buffer. (B) Localization of the copper resistance gene cluster of *X. axonopodis* pv. *vesicatoria* XvP26 to genomic DNA of the bacterium by Southern hybridization. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% Seakem-GTG agarose in 0.5X TBE buffer. The ramped pulse times were 5-45 s for 22 hr at 14 °C and 200 V. The blot was probed with the labelled 27.9-kb pLAFR3 clone carrying copper resistance genes.

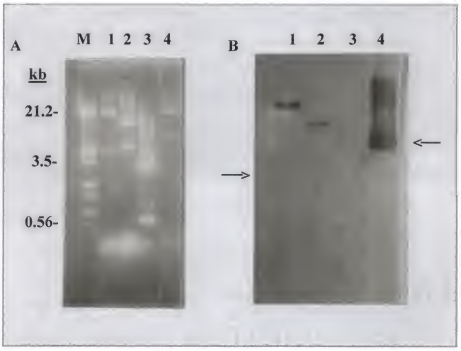

Fig. 3-7. Similarity among the plasmid-borne copper resistance genes from *X. axonopodis* pv. *vesicatoria* (75-3) and *Pseudomonas syringae* pv. *tomato* (pCOP35), and chromosomal-borne copper resistance genes from *X. arboricola* pv. *juglandis* (pXjCu99) probed with a subclone of copper resistance genes of *X. axonopodis* pv. *vesicatoria* XvP26. (A) Agarose gel electrophoresis of the copper resistance clones. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III; 1, copper resistance clone from 75-3 digested with *Eco*RI-*Hind*III; 2, copper resistance clone from pXjCu99 digested with *Cla*I; 3, copper resistance clone from pCOP35 digested with *Eco*RI-*Hind*III; 4, copper resistance genes from Xvp26 digested with *Eco*RI-*Hind*III. (B) Southern blot hybridization of the 10.9-kb *Eco*RI-*Hind*III fragment carrying copper resistance genes of the subclone probe to DNA from panel A. Arrows on left of panel B point to bands in lanes 1, 2, 3, that hybridized weakly, and arrows on right points to lane 4 that hybridized strongly with the probe. Washes were done with 0.5X SSC at room temperature.

polymorphism. We concluded that the cloned copper resistance gene cluster of XvP26 is divergent at the sequence level with the other cloned plasmid-borne and chromosomal-borne copper resistance genes based upon Southern hybridization and restriction enzyme analyses.

However, the copper resistance gene cluster may have some general *copABCD* structure characterized in *P. syringae* pv. *tomato* (Mellano and Cooksey, 1988a; Mellano and Cooksey 1988b). Based on available data, the origin of the copper resistance genes is still unclear, even though copper resistance genes cloned from different plant pathogenic bacteria have some level of similarity. Horizontal gene transfer among bacteria may be involved in the evolution of the copper resistance genes. Horizontal transfer of chromosomal copper resistance genes from XvP26 both *in vitro* and *in planta* may support this idea.

The copper resistance genes cloned from XvP26 may be inducible by low levels of copper. In one screening the cosmid library of strain XvP26 for copper resistance genes, I failed to find a clone with copper resistance genes (data not shown). This may be related to the inducible feature of copper resistance genes (Mellano and Cooksey, 1988b). However, after amendment of 20 μg/ml copper sulfate into media containing tetracyline and rifamycin, the clones containing copper resistance genes were found in the cosmid library of strain XvP26. This includes that the copper resistance genes are expressed under the copper-inducible promoter (Mellano and Cooksey 1988b). Previously described copper resistance genes from *X. axonopodis* pv. *vesicatoria* are plasmid-borne and are located on a self-transmissible plasmid about 200-188 kb in size (Bender et al.,

1990; Stall et al., 1986) and on a non-mobilizable plasmid 100 kb in size (Cooksey et al., 1990).

To localize copper resistance genes on the chromosome of the bacterium, we separated DNA fragments digested with a rare-cutting enzyme, *Spe*I, by PFGE. The copper resistance genes were localized with Southern hybridization using copper gene cluster from XvP26 as a probe. DNA fragments were separated in the range of 1200-200 kb in size by 5-45 s pulse time running program (Fig. 3-6A), and the copper genes were localized on about 500 kb chromosomal DNA fragment (Fig. 3-6B). There was no hybridization signal on the DNA fragments in the range of 200-6.5 kb in size separated by PFGE and *Spe*I using 1-13 s pulse time in running program. Based upon the results obtained in this part of the experiment, we conclusively showed that the copper gene cluster from strain XvP26 is located on the chromosome of the bacterium and the clone can be used as probe to detect horizontal transfer of the copper resistance genes.

In conclusion, the copper gene cluster from XvP26 is a unique chromosomal gene cluster based upon Southern hybridization in low stringency conditions.The hybridization signal between the gene cluster and plasmid-borne copper genes from *X. axonopodis* pv. *vesicatoria* was weak in low-stringency conditions. Understanding the structure, function and mechanism of copper resistance genes from different organisms, and characterization of additional copper genes from different organisms may help to improve knowledge on evolution and origin of copper resistance in plant pathogenic bacteria. This

knowledge may contribute the development of new strategies to control copper-resistant plant pathogenic bacteria in the field.

CHAPTER 4
DETERMINATION OF HORIZONTAL CHROMOSOMAL GENE TRANSFER AMONG STRAINS OF *Xanthomonas axonopodis* pv. *vesicatoria* BY CONJUGATION *IN PLANTA*

Introduction

The natural occurrence of gene transfer is important for adaptation of bacteria to a variety of ecological environments, and plays a vital role in the evolution of bacteria (Thomas and Helinski, 1989; Land and Reeves, 1996).

Strains of *X. axonopodis* pv. *vesicatoria*, which cause the bacterial spot disease of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) have great diversity in plasmid content (Canteros et al., 1995); most of the avirulence genes (Minsavage et al., 1990a) and genes for resistance to copper and streptomycin are located on plasmids (Stall et al., 1986; Minsavage et al., 1990b). Mobilization of plasmids among strains of *X. axonopodis* pv. *vesicatoria* occurs in nature (Canteros et al., 1995). However, there is no report of horizontal chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria* in nature.

The objective of this work is to investigate the presence of chromosomal gene transfer by conjugation in pepper leaves, and to determine if chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria* is affected by factors provided by the plant host.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study and their sources are listed in Appendix. All strains of *X. axonopodis* pv. *vesicatoria* were grown on nutrient agar (Becton Dickinson, Cockeysville, MD). Nutrient broth cultures (NB) were grown 24 hr on a rotatory shaker (150 rpm) at 28 ^{0}C. Conjugations between strains were performed on nutrient-yeast-glycerol agar (NYGA). All strains were stored in sterile tap-water at room temperature or in 30% glycerol at -70^{0}C, or both. Antimicrobial agents were used to maintain selection for resistance markers at the following final concentrations: Rifamycin, 80 μg/ml; nalidixic acid, 50 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; and copper sulfate, 200 μg/ml.

Plant Material and Plant Inoculations

The pepper cultivar Early Calwonder (ECW) was maintained in a growth chamber at 28-30 ^{0}C during inoculation and incubation. Leaves of pepper plants were inoculated with bacterial suspensions by infiltrating the bacteria into the mesophyll spaces of leaves with a 1 ml plastic syringe with a 27-gauge needle. The concentrations of bacteria in suspension were approximately 10^9 colony forming units (CFU) per milliliter in sterile tap-water, determined by measuring the optical density in a spectrophotometer (Bausch and Lomb, Inc., Rochester, NY).

Conjugation and Determination of Optimal Mating Time for Chromosomal Gene Transfer in planta

Both donor and recipient strains were grown on nutrient agar containing proper antimicrobial agents for overnight at 28 ^{0}C. Bacteria were collected from solid media, suspended in 1 ml sterile tap-water, washed two times by centrifugation, and resuspended in sterile tap-water. Donor (10^9 cfu/ml) and recipient (10^9 cfu/ml) cells were mixed very well by pipetting, infiltrated into mesophyll cells of pepper leaves, and incubated for 16, 24, 32, 40 and 48 hr at 28 ^{0}C. As a comparison, the same bacterial mixtures were incubated on nutrient-yeast-glucose agar (NYGA) for 16, 24, 32, 40 and 48 hr. Three cm^2 of inoculated leaf tissue were triturated in 1 ml sterile tap-water, vortexed, and pelleted at 14,000 rpm for 2 min, washed in 1 ml of sterile tap-water followed by centrifugation at 14,000 rpm for 2 min. The pellets were resuspended in 800 µl sterile tap-water and vortexed. All of each 800 µl bacterial suspension, which contained some leaf tissue, was spread onto two plates of media (400 µl for each plate) containing copper (200 µg/ml), rifamycin (80 µg/ml), nalidixic acid (50 µg/ml) and chlorothalonil (80 µg/ml), or kanamycin (50 µg/ml), rifamycin (80 µg/ml), nalidixic acid (50 µg/ml) and chlorothalonil (80 µg/ml), depending upon donor strain. Chlorothalonil was added to prevent growth of fungi present in leaf tissue. Plates were kept at 28 ^{0}C for 3-4 days. Numbers of conjugants were counted, and compared with results obtained *in vitro*.

Obtaining *hrp*- and *pig*- of The Recipient Strain of *Xanthomonas axonopodis* pv. *vesicatoria*

Bacteria were grown in 4 ml NB to the mild-logarithmic stage ($5x10^8$ cells/ml). The culture was centrifuged for 2 min at 14,000 rpm to pellet the cells, and the cells were resuspended in equal volume of the Tris-maleic acid buffer at pH 6.0 (Carlton and Brown, 1981). A final concentration of 100 μg/ml of a freshly prepared solution of nitrosoguanidine (NTG) was added to culture of the recipient strain and incubated at 28-30 ^{0}C without shaking for 40 min (50% of cell were killed). The treated culture was centrifuged as before, resuspended in sterile tap-water, plated on media containing rifamycin and nalidixic acid, and incubated at 28-30 ^{0}C. White colonies (R^{pig-}) were selected and tested for mutation in the pigmentation gene cluster by complementation experiments. A clone in pLAFR3 (EC744) containing the pigmentation genes (Minsavage, unpublished) was used in triparental mating with white colonies. A strain that was complemented to yellow color used as a recipient strain in chromosomal transfer experiments.

To obtain *hrp*$^-$ recipient strain, individual colonies selected after NTG treatment described above were inoculated into mesophyll cells of the pepper leaves in the concentration of about 10^8 cfu/ml. Plants were kept at 28-30 ^{0}C for 3 days to observe disease development. Selected non-pathogenic colonies were tested for proper mutation by complementation experiments. A clone in pLAFR3 containing the *hrp* genes (EC993) was used in triparental matings with non-pathogenic colonies. Transconjugants from triparental mating were reinoculated into pepper leaves to detect for complementation to pathogenicity. A complemented strain was selected as a *hrp*$^-$ recipient for further conjugation tests.

Plasmid Extraction

See in Chapter 2.

Preparation of Genomic DNA in Agarose Blocks

See in Chapter 2.

Restriction Endonuclease Digestions

See in Chapter 2.

Pulsed-Field Gel Electrophoresis

See in Chapter 2.

Southern Hybridization Analysis Using Large Fragments from PFGE Gels

See in Chapter 2.

Results

Growth of Donor, Recipient and Conjugants on Antibiotic Media

Donor Cu^r Str^r, recipient Rif^s Nal^r and putative conjugants (from conjugation performed in pepper leaves) Cu^r Rif^s Nal^r were tested for growth on antimicrobial media. The donor strain was able to grow on media containing copper and streptomycin, but not on media containing rifamycin, nalidixic acid, and copper, rifamycin and nalidixic acid (Fig. 4-1). The recipient strain grew on media containing rifamycin and nalidixic acid, but not copper and streptomycin, and copper, rifamycin and nalidixic acid (Fig. 4-1). Putative conjugants grew on

media containing rifamycin and nalidixic acid, and copper, rifamycin and nalidixic acid, but not media containing copper and streptomycin (Fig. 4-1).

Similarly, donor $Kn^r Str^r$, recipient $Rif^s Nal^r$ and putative conjugants $Kn^r Rif^s Nal^r$ (from conjugation performed in pepper leaves) were tested for growth on antimicrobial media. The donor strain grew on media containing kanamycin and streptomycin, but not media containing rifamycin and nalidixic acid, and kanamycin, rifamycin, nalidixic acid (Fig. 4-2). The recipient strain grew on media containing rifamycin and nalidixic acid, but not on kanamycin and streptomycin, and kanamycin, rifamycin and nalidixic acid (Fig. 4-2). The putative conjugants grew on media containing rifamycin and nalidixic acid, and kanamycin, rifamycin and nalidixic acid, but not on kanamycin and streptomycin (Fig. 4-2).

Plasmid Profiles of Donor, Recipient and Conjugants

The donor $Cu^r Str^r$ strain has a 15 kb plasmid, and recipient $Rif^s Nal^r$ strain has three plasmids in size of about 120 kb, 44kb and 33 kb. Putative conjugants $Cu^r Rif^s Nal^r$ had exactly same plasmid profile as recipient. A plasmid 15 kb in size was not detected in plasmid profile of conjugants (Fig. 4-3).

The donor $Kn^r Str^r$ strain has two plasmids in size about 300 kb and 40 kb. The recipient $Rif^s Nal^r$ strain has three plasmids in size of about 120 kb, 44 kb and 33 kb. Conjugants $Kn^r Rif^s Nal^r$ had exactly the same plasmid profile with recipient in size of about 120 kb, 44 kb, and 33 kb. Any plasmid in size of about 300 kb was not detected in plasmid profile of the conjugants (Fig. 4-4).

Horizontal Transfer of Copper Genes and Tn5 Sequences

The total genome profiles of DNA fragments of donor, recipient and putative conjugants were determined by PFGE and a rare-cutting enzyme, *Spe*I. Putative conjugants obtained from mating of donor $Cu^r Str^r$ and recipient Rif^r Nal^r mainly shared a genome profile with recipient strain except for some polymorphism, but conjugants were not similar to donor strain (Fig. 4-5, 4-6).

Similarly, putative conjugants obtained from mating of donor $Kn^r Str^r$ and recipient $Rif^r Nal^r$ mainly shared similar genome profile with the recipient except for some polymorphism, but putative conjugants showed different bands than from donor strain (Fig. 4-7, 4-8).

Copper resistance genes were located on both donor $Cu^r Str^r$ and conjugants' DNA fragments obtained by restriction of genomic DNA with *Spe*I and separated by PFGE in Southern hybridization tests (Fig. 4-9). Kanamycin resistance (Tn*5*) genes were located on DNA fragments of both donor $Kn^r Str^r$ and conjugants $Kn^r Rif^r Nal^r$ (Fig. 4-10).

Determination of Optimal Mating-Time for Conjugation Performed in Pepper Plants

The frequency of conjugation in pepper leaves increased with increasing mating-time from 16 hr to, 24, 32, 40 and 48 hr (Table 4-1). Frequency of chromosomal transfer of both copper and kanamycin resistance genes from donor to recipient strain was significantly higher than that obtained *in vitro* (Fig. 4-11, 4-12, 4-14). In 48-hr mating time, four conjugants were obtained *in vitro*.

However, more than 300 conjugants were obtained from 48-hr mating performed in pepper leaves. Although there were no conjugants detectable after 16 hr to 40 hr mating *in vitro*, the conjugants were detectable from 16 hr-mating *in planta*.

The relationship between numbers of conjugants and mating time was not logarithmic (Fig. 4-13) under these experimental conditions.

Horizontal Transfer of Pigmentation Genes

A mating of the kanamycin resistant donor in the mutant of the recipient that was deficient in production of pigment was performed in pepper leaves. Ninety percent of the conjugants (from 400 conjugants) were yellow. Therefore, horizontal transfer of chromosomal pigmentation (*pig*) genes occurred from donor to recipient.

Conjugants Kn^r Rif^s Nal^r *pig*$^+$ obtained from mating of donor Kn^s Str^r*pig*$^+$ and recipient Rif^s Nal^r *pig*$^-$ (Fig. 4-15) were tested for growth on antibiotic media. The donor strain was able to grow on media containing kanamycin and streptomycin, but not on media containing rifamycin and nalidixic acid, and kanamycin, rifamycin and nalidixic acid but not media containing kanamycin and streptomycin.

Ten conjugants were randomly selected and had exactly the same plasmid profile as the recipient, in size. Plasmids of about 300 kb and 40 kb (donor strain has these plasmids) were not detected in the plasmid profiles of the conjugants.

In the investigation of total genome profiles of donor, recipient and conjugants Kn^r Rif^s Nal^r *pig*$^+$ mainly shared similar genome profiles with the recipient

except for some polymorphism, but were not similar to the donor strain (Fig. 4-16, 4-17). DNA fragments were obtained from donor, recipient and conjugant by restriction of genomic DNA with *Spe*I and separated by PFGE. Tn*5* sequences were detected by Southern hybridization tests on DNA fragments of the donor and conjugant, but were not detected in the recipient. Kanamycin (Tn*5*) resistance genes and pigmentation genes were located on the same DNA fragment of the donor and conjugants (Fig 4-18, 4-19).

Horizontal Transfer of *hrp* Genes

A mating of the kanamycin resistant donor with a mutant of the recipient, deficient in pathogenicity factors, was performed in pepper leaves. Conjugants $Kn^r Rif^s Nal^r$ were confirmed by plasmid profiles, PFGE fragments, and Southern hybridization by using Tn*5* sequences as probe (Fig. 4-24). A group of conjugants were inoculated into pepper leaves to determine if horizontal transfer of *hrp* genes from the donor to the recipient strain occurred. Horizontal transfer of chromosomal *hrp* genes occurred in 5% of 400 conjugants as determined by complementation of hrp^- recipient strain into hrp^+ recipient strain which produces disease symptom (Fig. 4-20). Conjugants $Kn^r Rif^s Nal^r hrp^+$ from mating of donor $Kn^r Str^r hrp^+$ with recipient $Rif^s Nal^r hrp^+$ were tested for growth on antibiotic media. The donor strain was able to grow on media containing kanamycin and streptomycin, but not on media containing rifamycin and nalidixic acid, or kanamycin and streptomycin.

Fig. 4-1. Growth of donor $Cu^r Str^r$, recipient $Rif^s Nal^r$ and conjugant $Cu^r Rif^s Nal^r$ on antibiotic media. A, B, C: Growth of donor, recipient and conjugant on medium containing copper and streptomycin, respectively. D, E, F: Growth of donor, recipient and conjugant on medium containing rifamycin and nalidixic acid, respectively. G, H, I: Growth of donor, conjugant and recipient on medium containing copper, rifamycin and nalidixic acid, respectively.

Fig. 4-2. Growth of donor $Kn^r Str^r$, recipient $Rif^s Nal^r$ and conjugant $Kn^r Rif^s Nal^r$ on antibiotic media. A, B, C: Growth of donor, recipient and conjugant on medium containing kanamycin and streptomycin, respectively. D, E, F: Growth of donor, recipient and conjugant on medium containing rifamycin and nalidixic acid, respectively. G, H, I: Donor, recipient and conjugant on medium containing kanamycin, rifamycin and nalidixic acid, respectively.

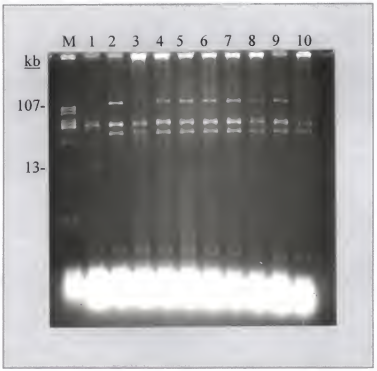

Fig. 4-3. Agarose gel electrophoresis of plasmid DNA from donor, recipient and conjugants. Lanes: M, plasmids of *Erwinia stewartii*; 1, donor $Cu^r Str^r$; 2, recipient $Rif^s Nal^r$; 3-10, conjugants $Cu^r Rif^s Nal^r$.

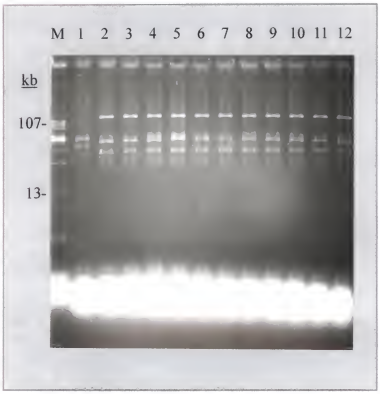

Fig. 4-4. Agarose gel electrophoresis of plasmid DNA from donor, recipient and conjugants. Lanes: M, plasmids of *Erwinia stewartii*; 1, donor $Kn^r Str^r$; 2, recipient $Rif^s Nal^r$; 3-12, conjugants $Kn^r Rif^s Nal^r$.

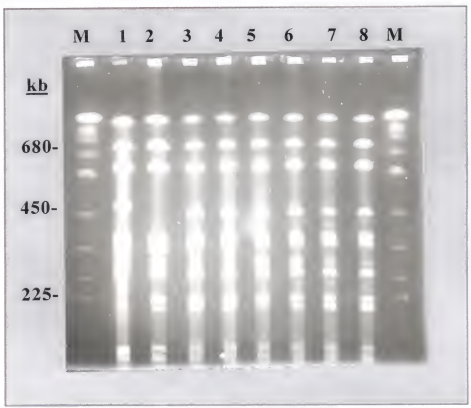

Fig. 4-5. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor Cu^r Str^r; 2, recipient Rif^s Nal^r; 3-8, conjugants Cu^r Rif^s Nal^r. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

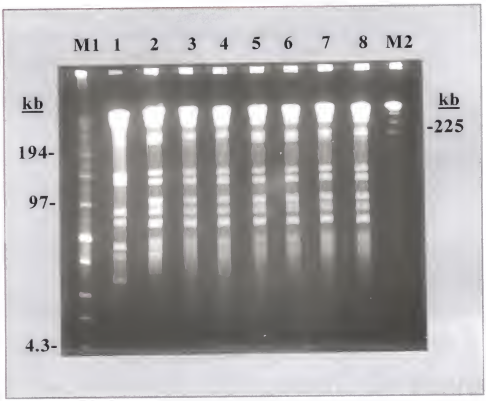

Fig. 4-6. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M1, Low-range PFGE marker; M2, DNA size standards of *Saccharomyces cerevisiae*; 1, donor Cu^r Str^r; 2, recipient Rif^s Nal^r; 3-8, conjugants Cu^r Rif^s Nal^r. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

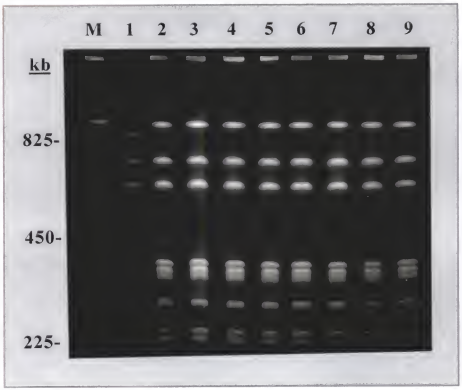

Fig. 4-7. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor $Kn^r Str^r$; 2, recipient $Rif^s Nal^r$; 3-9, conjugants $Kn^r Rif^s Nal^r$. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

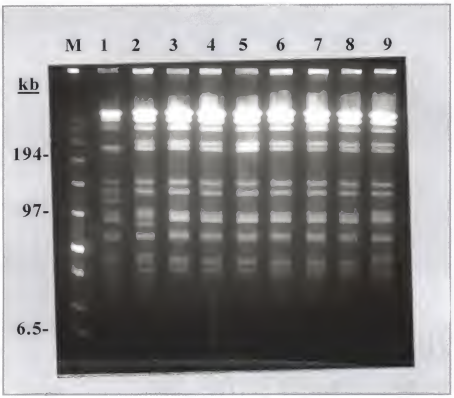

Fig. 4-8. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M, Low-range PFGE marker; 1, donor $Kn^r Str^r$; 2, recipient $Rif^s Nal^r$; 3-9, conjugants $Kn^r Rif^s Nal^r$. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

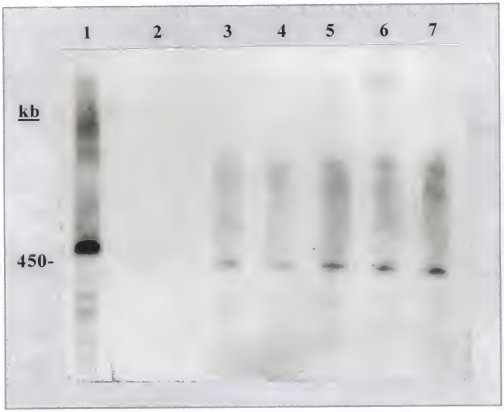

Fig. 4-9. Hybridization of a clone carrying copper gene cluster of *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: 1, donor Cu^r Str^r; 2, recipient Rif^s Nal^r; 3-7, conjugants Cu^r Rif^s Nal^r. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 V. The clone containing copper resistance genes was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

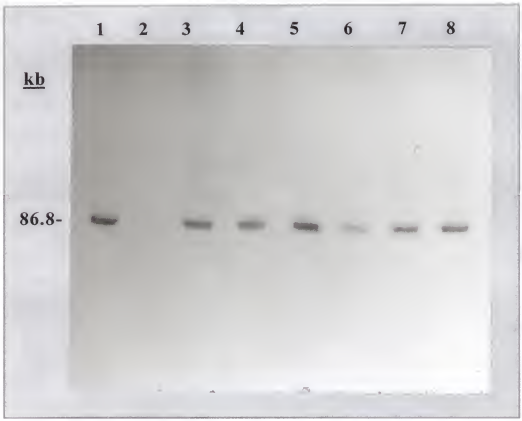

Fig. 4-10. Hybridization of a clone carrying Tn*5* sequences *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: 1, donor Km^r Str^r; 2, recipient Rif^s Nal^r; 3-8, conjugants Km^r Rif^s Nal^r. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. Tn*5* sequences (2.96 kb) was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

Table 4-1. Optimal mating-time for transfer of chromosomal Tn*5* sequences from donor to recipient strain of *X. axonopodis* pv. *vesicatoria in planta*.

Mating-time (hour)	Frequency of conjugation (per donor)
0	0
8	0
16	1.5×10^{-7}
24	4.72×10^{-7}
32	8.75×10^{-7}
40	1.99×10^{-6}
48	2.65×10^{-6}

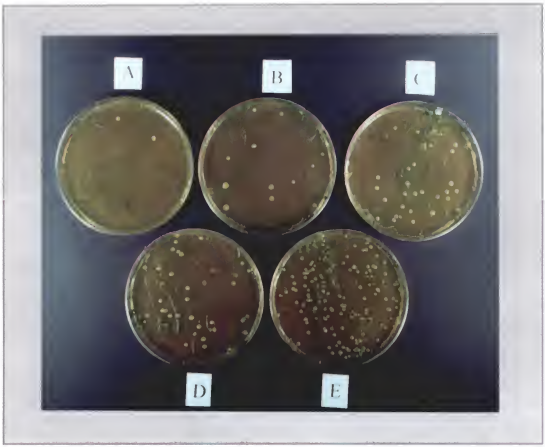

Fig. 4-11. The effect of mating time on conjugation in pepper leaves. A, B, C, D and E, conjugants Kn^r Rif^s Nal^r from mating times 16 hr, 24 hr, 32 hr, 40 hr and 48 hr, respectively. Conjugants were obtained on media containing kanamycin, rifamycin, nalidixic acid and chlorothalonil.

Fig. 4-12. The effect of mating time on conjugation in NYGA medium. A, B, C, D and E, conjugants Kn^r Rif^s Nal^r from mating times 16 hr, 24 hr, 32 hr, 40 hr and 48 hr, respectively. Conjugants were obtained on media containing kanamycin, rifamycin, nalidixic acid and chlorothalonil.

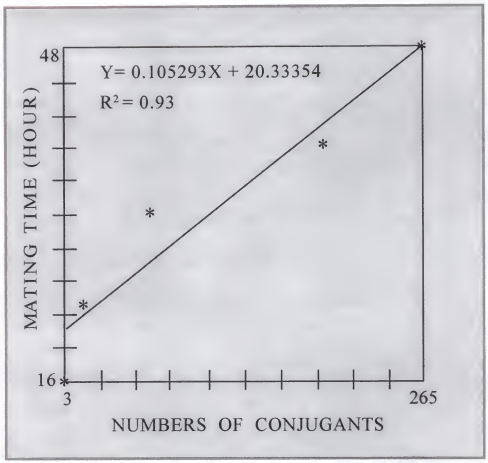

Fig. 4-13. The relationship between the mating time and the numbers of conjugants $Kn^r Rif^s Nal^r$ obtained from conjugation in pepper leaves.

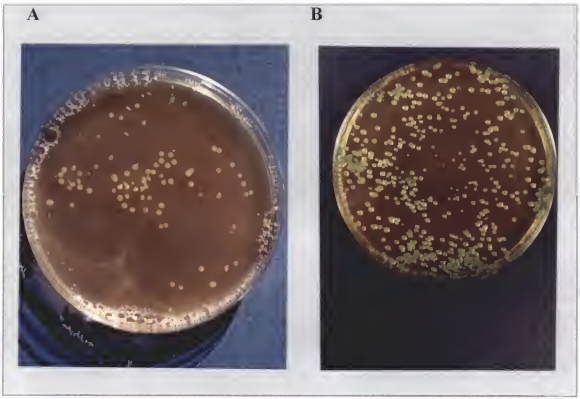

Fig. 4-14. Conjugants obtained from mating performed on NYGA and pepper leaves. (A), Conjugants $Cu^r Rif^s Nal^r$ from mating performed on NYGA medium for 48 hr were obtained on medium containing copper sulfate, rifamycin and nalidixic acid. Donor $Cu^r Str^r$ and recipient $Rif^s Nal^r$ were used in mating. (B), Conjugants $Cu^r Rif^s Nal^r$ from mating performed in pepper leaf for 48 hr. Donor $Cu^r Str^r$ and recipient $Rif^s Nal^r$ were used for mating.

Fig. 4-15. Complementation of white color recipient strain of *X. axonopodis* pv. *vesicatoria* into yellow color by conjugation performed in pepper leaf. (A), donor Kn^r Str^r pig^+; (B), recipient Rif^r Nal^r pig^-; (C), conjugants Kn^r Rif^r Nal^r pig^+.

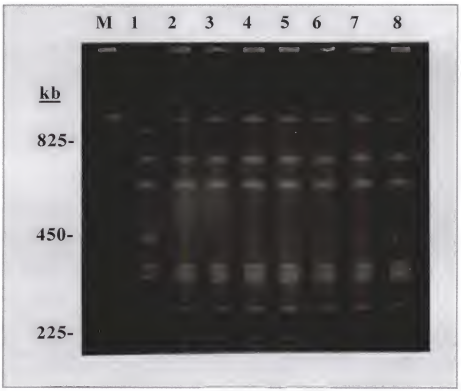

Fig. 4-16. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor Kn^r Str^r *pig*$^+$; 2, recipient Rif^r Nal^r *pig*$^-$; 3-8, conjugants Kn^r Rif^r Nal^r *pig*$^+$. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

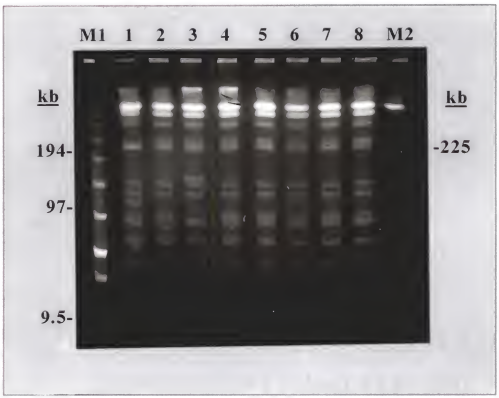

Fig. 4-17. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M1, Low-range PFGE marker; M2, DNA size standards of *Saccharomyces cerevisiae*; 1, donor Kn^r Str^r *pig*$^+$; 2, recipient Rif^s Nal^r *pig*$^-$; 3-8, conjugants Kn^r Rif^s Nal^r *pig*$^+$. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

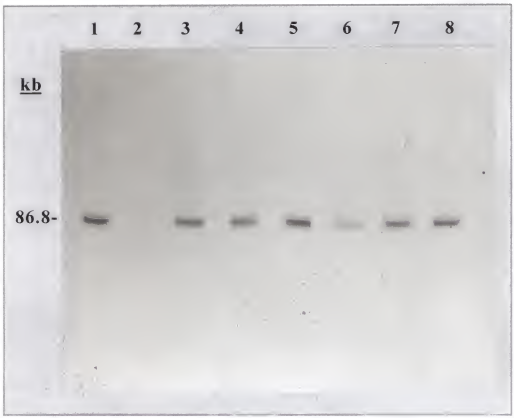

Fig. 4-18. Hybridization of a clone containing Tn*5* sequences from *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: 1, donor Km^r Str^r *pig*$^+$; 2, recipient Rif^s Nal^r *pig*$^-$; 3-8, conjugants Km^r Rif^s Nal^r *pig*$^+$. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulsed times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. Tn*5* sequences (2.96 kb) was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

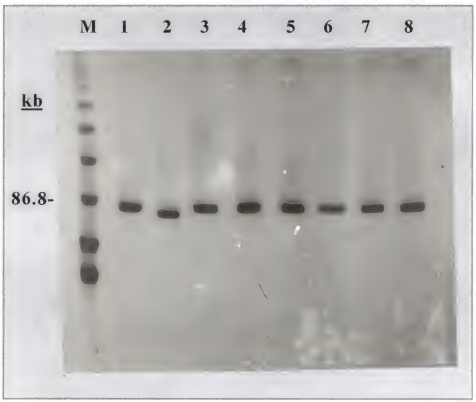

Fig. 4-19. Hybridization of a clone containing *pig* genes from *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: M, Low-range PFGE marker; 1, donor $Kn^{+}Str^{+}pig^{+}$; 2, recipient $Rif^{+}Nal^{+}pig^{-}$; 3-8, conjugants $Kn^{+}Rif^{+}Nal^{+}pig^{+}$. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. The clone containin *pig* genes were labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

Fig. 4-20. Complementation of *hrp*⁻ recipient strain of *X. axonopodis* pv. *vesicatoria* into *hrp*⁺ by conjugation performed in pepper leaf. (A), donor Km^r Str^r *hrp*⁺; (B), recipient Rif^s Nal^r *hrp*⁻; (C), conjugant Km^r Rif^s Nal^r *hrp*⁺; (D), conjugant Km^r Rif^s Nal^r *hrp*⁻.

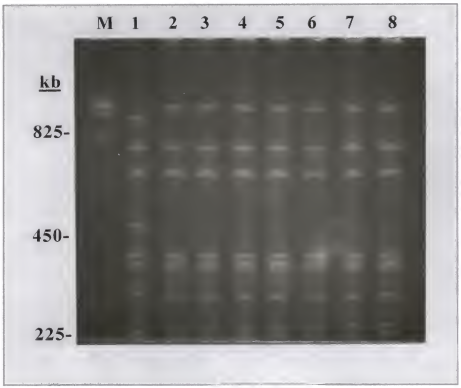

Fig. 4-21. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M, DNA size standards of *Saccharomyces cerevisiae*; 1, donor Kn^r Str^r *hrp*$^+$; 2, recipient Rif^s Nal^r *hrp*$^-$; 3-8, conjugants Kn^r Rif^s Nal^r *hrp*$^+$. The ramped pulse times were 5-45 s for 22 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

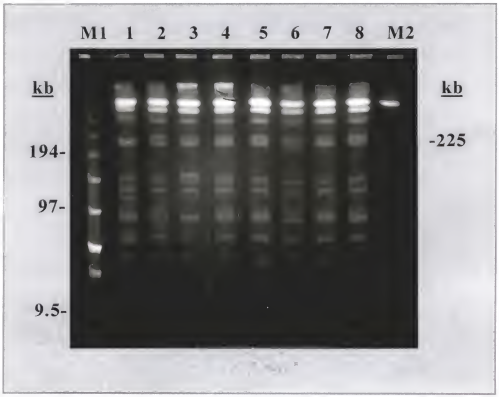

Fig. 4-22. PFGE of *Spe*I digests of genomic DNA of donor, recipient and conjugants from mating performed in pepper leaves for 48 hr. Lanes: M1, Low-range PFGE marker; M2, DNA size standards of *Saccharomyces cerevisiae*; 1, donor Kn^r Str^r *hrp*$^+$; 2, recipient Rif^s Nal^r *hrp*$^-$; 3-8, conjugants Kn^r Rif^s Nal^r *hrp*$^+$. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. The gel was 0.9 % Seakem-GTG agarose in 0.5X TBE buffer.

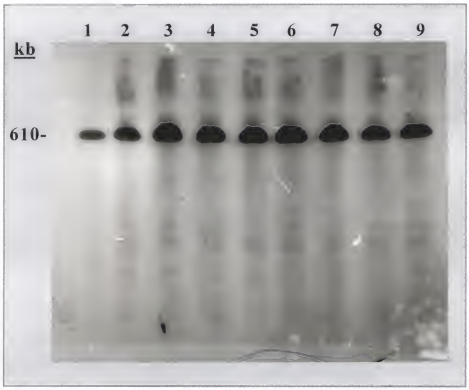

Fig. 4-23. Hybridization of a clone containing *hrp* genes from *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: 1, donor Km^r Str^r hrp^+; 2, recipient Rif^s Nal^r hrp^-; 3-9, conjugants Km^r Rif^s Nal^r hrp^+. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulse times were 5-45 s for 22 hr at 14 °C and 200 V. The clone containing *hrp* genes was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

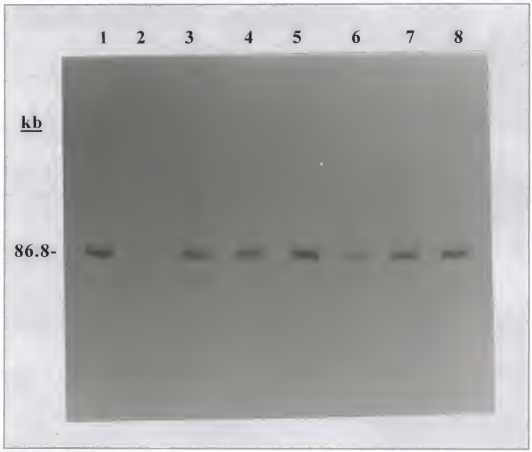

Fig. 4-24. Hybridization of a clone containing Tn*5* sequences from *X. axonopodis* pv. *vesicatoria* to genomic DNA of donor and conjugants. Lanes: 1, donor Kn^r Str^r *hrp*$^+$; 2, recipient Rif^s Nal^r *hrp*$^-$; 3-8, conjugants Kn^r Rif^s Nal^r *hrp*$^+$. Total genomic DNA was restricted with *Spe*I and separated by PFGE in 0.9% agarose gel. The ramped pulse times were 1-13 s for 12 hr at 14 ^{0}C and 200 V. Tn*5* sequences (2.96 kb) was labeled by random-primed incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Non-Radioactive Chemiluminescent DNA Labeling and Detection Kit.

Conjugants $Kn^rRif^rNal^r$ had exactly the same plasmid profile as recipient. Plasmids of about 300 kb and 40 kb (donor strain has these plasmids) were not detected in the conjugants.

In the investigation of total genome profiles of donor, recipient and conjugants $Kn^rRif^rNal^r$ *hrp*$^+$ mainly shared similar genome profiles with the recipient except some polymorphism, but were not similar to donor strain (Fig. 4-21, 4-22).

Kanamycin (Tn*5*) resistance genes were detected on both donor and conjugants' DNA fragments (86.8 kb) obtained by restriction of genomic DNA with *Spe*I and separated by PFGE in Southern hybridization tests (Fig. 4-24). On the other hand, *hrp* genes were located on 610 kb DNA fragments of donor, recipient and conjugants by Southern hybridization using *hrp* genes as probe (Fig. 4-23).

Discussion

Conjugation frequency in pepper leaves was significantly higher than that *in vitro* (Chapter 2) (Table 4-1) (Fig. 4-11, 4-12, 4-13, 4-14). Increased numbers of conjugants occurred up to 48 hr in pepper leaves. The maximum number of conjugants were obtained after 72 hr mating *in vitro*, however. The significant increase (more than 100 times) in the frequency of chromosomal transfer of copper and Tn*5* sequences in pepper leaves may be explained by involvement of some plant factors in conjugation.

In addition to using antibiotic and copper resistance markers encoded by

chromosomal genes in strains of *X. axonopodis* pv. *vesicatoria*, plasmid profiles and fragment profiles of genomic DNA of donor, recipient and conjugants were used to confirm the transfer of chromosomal DNA from donor to recipient. Attention was focused on the presence of horizontal transfer of cloned chromosomal genes; copper resistance (*cop*), hypersensitive reaction and pathogenicity (*hrp*), pigmentation (*pig*) and kanamycin resistance (Tn*5* sequences) from two donor strains to a recipient strain of the bacterium by conjugation performed in pepper leaves, a natural niche of the bacterium.

The evidence for horizontal transfer of *cop* genes and Tn*5* sequences under these experimental conditions provoked the question of whether or not transfer could happen for other chromosomal genes from one strain to another strain in nature.The increased numbers of conjugants obtained by matings *in planta* allowed the investigation of linkage of copper resistance and kanamycin resistance to other genes. For this, I examined horizontal transfer of *hrp* genes and *pig* genes. All conjugants from mating of donor Km^r Str^r hrp^+pig^+ and the recipient strains that were hrp^- and pig^- were confirmed by plasmid profiles, PFGE fragments, and Southern hybridization by using Tn*5* sequences as a probe. Horizontal transfer of chromosomal *hrp* genes occurred in 5% of the total conjugants. On the other hand, horizontal transfer of *pig* genes occurred in 90% of total conjugants. These results suggested that when Tn*5* sequences (kanamycin resistance) were transferred from donor to recipient hrp^- and pig^- strains, horizontal transfer of *hrp* and *pig* genes also occurred, but their transfer frequency was different from each other. This could be explained by the different distances of the *hrp* and *pig* loci from the Tn*5* genetic marker on the chromosome of the donor strain. This idea was confirmed with results of Southern hybridization

tests for fragments from PFGE analysis with probes of Tn*5*, *hrp* genes and *pig* genes. Tn*5* sequences and *pig* genes were located on the same *Spe*I-restricted fragment separated by PFGE (Fig. 4-18, 4-19), and Tn*5* and *hrp* genes were located on differents fragments (Fig. 4-23, 4-24). These results suggested that loci of *pig* genes and Tn*5* sequences are very close to each other, but *hrp* genes are not as close as *pig* genes to Tn*5* genetic marker on chromosome of the donor strain $Kn^r Str^r$. These findings were in good agreement with results obtained from recently constructed physical genome map of the bacterium that showed the location of *pig* genes and *hrp* genes on the chromosome of the bacterium (Hacioglu et al., 1996).

We concluded that horizontal transfer of chromosomal genes; *cop*, *hrp*, *pig* and Tn*5* sequences from donor to recipient strain of *X. axonopodis* pv. *vesicatoria*, and its occurrence *in planta* make possible the presence of the horizontal chromosomal gene transfer in nature. Although only two donor strains were used in these experiments, horizontal chromosomal gene transfer among *X. axonopodis* pv. *vesicatoria* may be common in nature.

CHAPTER 5

CLONING OF *tra* GENES FROM *Xanthomonas axonopodis* pv. *vesicatoria*

Introduction

Several *tra* gene clusters have been cloned from different bacteria. In *Escherichia coli, tra* genes were located within a 34-kb region of the F plasmid, and were involved in regulation of DNA transfer, synthesis of pili, aggregate stabilization, conjugal DNA metabolism and surface exclusion during conjugation (Ippen-Ihler and Skurray, 1993). *tra* genes were also characterized from the plant pathogenic bacterium, *Agrobacterium tumefaciens*. The genes are involved in conjugation of the Ti plasmid of *Agrobacterium tumefaciens*, and were located in three distinct regions of the pTiC58 plasmid, designated TraI, TraII and TraIII (Kado, 1993).

tra (transfer) genes that are involved in conjugal transfer of DNA should be present to facilitate horizontal chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria.* Determination of the presence of *tra* genes in the chromosome of donor strains of *X. axonopodis* pv. *vesicatoria* would provide further evidence for horizontal transfer of chromosomal genes. The objective of this work was to screen a DNA library of strain XvP26 for *tra* genes.

Materials and Methods

Bacterial Strains, Plasmid, and Culture Conditions

The bacterial strains and plasmids used in this study and their sources are listed in Appendix. The strains (85-13 and 82-8) of *X. axonopodis* pv. *vesicatoria* were grown on nutrient agar (Becton Dickinson, Cockeysville, MD). Nutrient broth cultures (NB) were grown 24 hr on a rotatory shaker (150 rpm) at 28 ^{0}C. Strains of *Escherichia coli* were cultivated on Luria-Bertani medium at 37 ^{0}C (Miller, 1972). Triparental matings were performed on nutrient-yeast-glycerol agar (NYGA). All strains were stored in sterile tap-water at room temperature or in 30% glycerol at 70 ^{0}C, or both. Antibiotics were used to maintain selection for the resistance marker at the following final concentrations: Tetracycline, 10 μg/ml; rifamycin, 80 μg/ml; kanamycin, 50 μg/ml.

General DNA Manupulations

Miniscale preparations of *E. coli* plasmid DNA were made by an alkaline lysis method as described by Sambrook et al. (1989). The restriction endonuclease digestions were carried out according to conditions specified by the manufacturer. The restricted DNAs were separated by electrophoresis in 1% agarose gel (Seakem GTG, FMC Bioproduct, Rockland, ME) in TAE buffer at 5V/cm. The gel was stained with 0.5 μg of ethidium bromide per ml for 30 min, and then photographed over a UV transluminator with 55 polaroid film.

Bacterial Conjugation and Isolation of Clones of *tra* Genes

A library of DNA fragments in the cosmid pLAFR3 from strain XvP26 was obtained from G.V. Minsavage (University of Florida, Gainesville). There were 1100 clones in the library. Each cosmid was transferred to cells of strain 82.8 Rif^r by triparental matings (Figurski and Helinski, 1979) in which mid-log growth phase cells of 82-8 Rif^r of *X. axonopodis* pv. *vesicatoria* as the recipient were mixed with *E. coli* DH5α (cosmid clones) as the donor and HB101 (PRK2073) as the conjugational helper. The volume ratio of recipient-donor-helper was 2:1:1. The mixture was spread onto NYGA (Nutrient yeast glycerol agar) (Daniels et al., 1984) plates and incubated overnight at 28 ^{0}C. The conjugants were then selected on NA (Nutrient agar) medium containing rifamycin and tetracyline. Conjugants (single colonies) Rif^r Tet^r were transferred onto medium containing rifamycin and tetracyline for their purification and maintenance until they were used.

Conjugants (82-8 Rif^r Tet^r), each containing a clone, were transferred to NYGA medium and incubated overnight at 28 ^{0}C. Each plate had 20 bacterial spots. Cells of strain 85-13 Kn^r Str^r of *X. axonopodis* pv. *vesicatoria* from a 16-20 hr-old culture were also inoculated as small drops with very small loop on NYGA plates for overnight. Each overnight-growth colony (82-8 Rif^r Tet^r carrying cosmid clone) was mixed with an overnight- growth colony of strain 85-13 Kn^r Str^r in equal volume with a sterile toothpick and incubated at 28 ^{0}C overnight. Bacteria from each mixture were spread onto medium containing kanamycin and tetracyline, and incubated at 28 ^{0}C for 3-4 days to detect conjugants.

Results

Cloning of *tra* Genes from *X. axonopodis* pv. *vesicatoria* XvP26

Of 1100 clones of the cosmid library of strain XvP26 90 % of were successfully transferred to cells of strain 82-8 Rif^r from *E. coli* by triparental mating. In the second mating, the 82-8 Rif^r Tet^r cultures were mated with the recipient strain 85-13 Kn^r Str^r. Only three clones resulted in conjugants. One of the clones was selected to use in further experiment, because it was the most consistent in terms of mobilization between strains. However, mobilization of the pLAFR3 clone between the strains of *X. axonopodis* pv. *vesicatoria* was not consistent in all experiments.

The digestion of cloned plasmid DNA with *Eco*RI and *Hind*III resulted in 3 fragments and 15-kb in size total inserted DNA (Fig. 5-1).

Discussion

The cosmid pLAFR3 does not have *tra* genes and is not self-transmissible. The cosmid does have *mob* genes and can be mobilized between strains of bacterial with *tra* genes from another plasmid. The helper strain HB101 (pRK 2073) supplies the plasmid in the *tra* genes in the triparental matings. If an inserting pLAFR3 contained *tra* from *X. axonopodis* pv. *vesicatoria*, then the cosmid could be self-transmissible between strains of the plant pathogen.

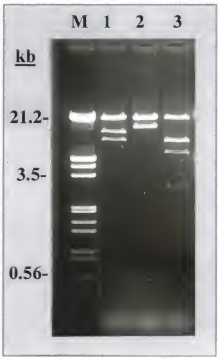

Fig. 5-1. Restriction endonuclease fragments of the cosmid clone carrying *tra* gene cluster from *X. axonopodis* pv. *vesicatoria* XvP26. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III; 1, 2, 3 cosmid clone carrying *tra* genes digested with *Eco*RI, *Hind*III and *Eco*RI-*Hind*III, respectively.

Clones in a library of strain XvP26 were found that had inserts that cause pLAFR3 to be self-transmissible. Presumably the insert had the *tra* genes necessary for this characteristic, but mobilization of the clones between strains of *X. axonopodis* pv. *vesicatoria* was inconsistent. Perhaps all genes necessary for the transfer function may not be present in the cloned 15 kb DNA fragment, so that consistent transfer of the cosmid from donor to recipient strain may not occur. *tra* genes of the F plasmid are located in 34 kb of the transfer region (Ippen-Ihler and Skurray, 1993). Therefore, since some of the *tra* genes may be missing, because of small size of the insert on DNA, the clone sometimes may have failed to transfer. It may be useful to find other related clones from cosmid library of the bacterium by the same technique, colony hybridization techniques using the cloned genes, or using the plant system. Conjugation between 82-8 Rif^r Tet^r and 85-13 Kn^r can be performed in pepper leaves, which may significantly increase the chance to find additional clones carrying *tra* genes from the cosmid library of the bacterium. The transfer frequency of chromosomal genes among the strains *in planta* was significantly higher than that obtained *in vitro*.

The origin of the transfer (*oriT*) region may be on the same clone. On many plasmids, the *oriT* region is close to *tra* genes and located at the end of the segment that includes *tra* genes (Wilkins and Lanka, 1993). The DNA of a self-transmissible plasmid is nicked at the *oriT* region as the result of expression of the *tra* genes. Expression of *tra* genes can be either inserted DNA-borne or host-origin (*tra* of recipient) such that host chromosome or plasmids may have *tra* genes. However, considering juxtaposition of the both genes on many plasmid

DNAs, it is likely that self-mobilizable clones may have both genes.

Characterization of these genes, specifically *tra* genes, and sequencing, could be used to develop specific primers for amplification of the small region of the *tra* genes by PCR. Screening of strains of *X. axonopodis* pv. *vesicatoria* and even other plant pathogenic bacteria for presence of *tra* genes by PCR may help to determine origins of this ancient gene cluster in bacteria. This may also help to determine universality of chromosomal gene transfer among strains of *X. axonopodis* pv. *vesicatoria* and pathovars of *Xanthomonas*. This knowledge can contribute to better understanding of horizontal gene transfer and evolution of bacteria.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Bacteria in the genus *Xanthomonas* are a diverse group of bacteria that occur worldwide and cause disease on many plants. Strains of *Xanthomonas axonopodis* pv. *vesicatoria* that cause bacterial spot disease of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) also have a great diversity in terms of restriction fragment length polymorphism (Cooksey and Graham, 1989), and plasmid content (Canteros et al., 1995). Most of the avirulence genes involved in host-specificities as well as cultivar specificities (Minsavage et al., 1990a; Stall, 1993), and genes for resistance to copper and streptomycin are located on plasmids (Stall et al., 1986; Minsavage et al., 1990b). Moreover, plasmids that carry copper resistance genes are self-transmissible (Stall et al., 1986), and mobilization of plasmids among strains of *X. axonopodis* pv. *vesicatoria* occurs in nature (Canteros et al., 1995). Based upon this knowledge, plasmids may have profound impact on gene flow among cells of *Xanthomonas* in nature. Plasmids probably are the major vehicles for transfer of genes among strains of *X. axonopodis* pv. *vesicatoria*. However, this type of gene transfer may not explain the evidence obtained for horizontal transfer of *hrp* gene sequences among pathovars of *Xanthomonas* (Leite et al., 1994), unless horizontal chromosomal gene transfer occurs among pathovars, or strains of pathovars of the bacterium.

Evidence has been obtained in this research for the occurrence of chromosomal transfer among strains of *X. axonopodis* pv. *vesicatoria*. We focused our attention on horizontal transfer of cloned chromosomal genes; copper resistance (*cop*), hypersensitive reaction and pathogenicity (*hrp*), pigmentation (*pig*) and kanamycin resistance (Tn*5* sequences) from two donor strains to a recipient strain of bacterium by conjugations performed *in vitro* and in pepper leaves. Occurrence of chromosomal transfer of Tn*5* sequences and copper genes was first found *in vitro* in the frequency of 10^{-8} per donor cell.

Antibiotic resistance markers encoded by chromosomal genes on both donor and recipient strain, and examining plasmid profiles of donor, recipient and conjugants provided key opportunities for both detection of appropriate putative conjugants and comparison of backgrounds of conjugants with donor and recipient strains, respectively. However, occurrence of putative conjugants obtained from media containing copper, rifamycin and nalidixic acid, or kanamycin, rifamycin, nalidixic acid (depending upon donor strain used) were not strong evidence for chromosomal gene transfer from donor to recipient, even though plasmid profiles of conjugants were similar to the recipient strain. I have undertaken the strategy of Pulsed-Field Gel Electrophoresis (PFGE) of DNA fragments after digestion of total genomic DNA with a rare-cutting endonuclease to compare the total genomes of donor, recipient and putative conjugants and to examine horizontal chromosomal gene transfer. The assumption is if chromosomal DNA is transferred from donor to recipient, the transfer of non-homologous DNA would be expected to change restriction sites of the genome of the conjugants. This would be strong evidence for localization of the transferred DNA sequences on the chromosome of conjugants as well as donor strains.

A unique chromosomal copper gene cluster of one donor strain and Tn*5* sequences of another strain were used to determine chromosomal gene transfer among three strains that originally were from different geographical locations.

Major evidence for chromosomal gene transfer was obtained by PFGE. Copper and kanamycin resistance genes were conclusively demonstrated to occur on the chromosome. Demonstration of transfer of chromosomal copper and kanamycin resistance genes to a recipient *in vitro* gave rise to the possibility of the transfer of other chromosomal genes among the strains *in vitro*. However, detection of transfer of *hrp* and *pig* chromosomal genes from donor to recipient strain deficient in *hrp* and *pig* failed *in vitro*. It was thought that this might be related to the distance of their linkage with copper and Tn*5* selective markers on the chromosome of the donor strains. The low frequency of horizontal transfer of copper and Tn*5* sequences *in vitro* that may not be enough to detect conjugants in which recombination of *hrp* and *pig* genes occurred.

Conditions for increasing chromosomal gene transfer to a significant level to obtain more conjugants were thought to be needed for detection of transfer chromosomal genes; *hrp* and *pig*. Experiments of conjugation in pepper plants were thought to be the best of candidates for looking for optimal condition for chromosomal gene transfer. Possibly conjugation might be inducible by plant as in the *Agrobacterium*-plant system.

Based upon the results of growth on antibiotic media, plasmid profiles, total genome fragment profiles and Southern hybridization with Tn*5* sequences and copper resistance genes as probes, it was clearly demostrated that Tn*5*

sequences and copper resistance genes were transferred from donor to recipient in a significantly higher frequency (10^{-6} per donor) in pepper leaves. This is similar to the Ti plasmid of *Agrobacterium tumefaciens* which conjugate significantly more *in planta,* and opines produced in neoplastic cells induce plasmid transfer of *Agrobacterium* (Kerr et al., 1977; Genetello et al., 1977). Similarly, some compound or compounds in pepper leaves may be involved in induction of chromosomal gene transfer of *X. axonopodis* pv. *vesicatoria*.

Four hundred of conjugants were obtained after matings were performed in pepper leaves. I again examined the horizontal transfer of the chromosomal genes, *hrp* and *pig*. Horizontal transfer of *hrp* genes from donor Kn^r Str^r *hrp*$^+$ to recipient Rif^s Nal^r *hrp*$^-$ strain occurred in 5% of 400 conjugants. On the other hand, horizontal transfer of *pig* genes occurred in 90% of 400 conjugants from Kn^r Str^r *pig*$^+$ and recipient Rif^s Nal^r *pig*$^-$ mating as determined by complementation of white colonies to yellow colonies the natural color of the bacterium. These results suggested that when kanamycin resistance was transferred with the Tn*5* sequences from donor Kn^r Str^r *pig*$^+$ to recipient Rif^s Nal^r *pig*$^-$, horizontal transfer of *hrp* and *pig* genes also occurred. The differences in their transfer frequency may be explained by the distance of each locus from the Tn*5* genetic marker on the chromosome of the donor strain. Fragments obtained by PFGE were probed in Southern hybridization tests with Tn*5* and *pig* genes sequences. Tn*5* and *pig* genes were located on the same *Spe*I-restricted fragments, and Tn*5* and *hrp* genes located on different fragments. The loci for *pig* genes and Tn*5* sequences are very close to each other, but *hrp* genes are more distant than *pig* genes to the Tn*5* genetic marker on the chromosome of the donor.

Occurrence of horizontal transfer of chromosomal genes such as shown here for *hrp*, *pig*, Tn*5*, and *cop*, may explain the presence of the relatively significant diversity among strains of the bacterium in terms of restriction fragment length polymorphism (Cooksey and Graham, 1989). These results were in good aggreement with previous observations (Bonas et al., 1991; Stall and Minsavage, 1990) on the conservation of the *hrp* region among plant pathogenic bacteria, confirmed by amplifying small *hrp* fragments from *X. campestris* pv. *vesicatoria* 75-3 (Leite et al., 1994). Plasmid transfer among strains of this bacterium has been demonstrated to occur in nature. It would not be a great extension to belive that chromosomal gene transfer also occurs in nature.

The phenomenon of conjugation in bacteria provides one of the cornerstones of bacterial genetics (Willets, 1993). The discovery of chromosome mobilization by the F plasmid in *Escherichia coli* opened up the genetic analysis of the bacterial chromosome. Conjugative chromosome transfer systems were established in many bacterial genera and permitted the construction of chromosome maps (Reimmann and Haas, 1993). Extensive knowledge of chromosomal gene arrangement in bacteria is known (Holloway, 1993). Gene mapping in bacteria was orginally based on the ability of an externally derived genetically marked fragments of DNA to recombine with the homologous region of the resident DNA of a recipient (Drlica and Riley, 1990). Resulting genetic maps reveal relationships among genes such as operon clusters and show orientation preferences that may reflect chromosomal activities.

The demonstration of horizontal chromosomal gene transfer in this work opens many opportunities for characterization of the chromosome of a plant pathogen. The techniques designed for detection and verification of recombinant (conjugants) in this work will be a contribution for detailed molecular and genetic analysis of chromosome of the bacterium. Indeed, different recombination frequencies of *pig* and *hrp* genes in this work were fully supported by recently constructed physical and genetic map of the bacterium (Hacioglu et al., 1996). Construction of the complete genetic map of the bacterium would be possible if recombination frequencies of other cloned or known genes are determined and the results are combined with hybridization of gene clones on the restricted with rare-cutting enzymes and separated by PFGE.

Chromosomal transfer of all genes would be possible by obtaining insertions of Tn*5* sequences into different locations on the chromosome of the donor strain of *X. axonopodis* pv. *vesicatoria*. Many other genes can be mobilized and their recombination frequency can be determined as described transfer of *hrp* and *pig* genes.

It is possible that chromosomal gene transfer is mediated by an insertion of a plasmid into the chromosome. A plasmid would supply the *tra* genes necessary for conjugation. Horizontal chromosomal transfer occurred in this study without introduction of a plasmid that integrated into the chromosome. However, the presence of *tra* genes in the chromosome of *X. axonopodis* pv. *vesicatoria* may be the result of a stably integrated plasmid in the bacterium.

tra genes from the chromosome of the donor $Cu^r Str^r$ have been cloned and probing other strains with the cloned *tra* genes may determine the universality of horizontal chromosomal genes in the bacterium. The universality of the phenomenon cannot be assumed from this study, because only two donor strains were used in our experiments. However, the strains were from widely different geographic locations (donor $Cu^r Str^r$, Taiwan; donor $Kn^r Str^r$, Florida). If horizontal transfer of chromosomal genes is common in nature, much variability can be explained. In addition, chromosomal gene transfer across pathovars may explain the broad plant pathogenicity of *Xanthomonas* (Leyns et al., 1984; Hayward, 1993).

APPENDIX

BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Table. List of bacterial strains and plasmids used in molecular transformation and conjugation.

Strain	Relevant characteristics	Source[a] / source of reference
Bacteria		
Xanthomonas axonopodis pv. *vesicatoria*		
XvP26	Cu^r	RES
82-8	Rif^r	RES
85-13	Str^r	RES
75-3	has a large plasmid (284 kb)	RES
E3	has a large plasmid (213 kb)	RES
Erwinia stewartii SW2	has 13 plasmid in different size	DLC
Escherichia coli		
DH5α	F- recA 80dlacZM15	BRL
HB101	F- recA	BRL
RR1	F- $recA^+$	CK
ED8767	$recA^-$	RES
Plasmids		
pLAFR3	Tet^r rlx^+ RK2 replicon	Staskawicz et al., 1987
EC744	*pig*$^+$	RES
EC993	*hrp*$^+$	RES

Continued on following page

Table-Continued

Strain	Relevant characteristics	Source[a] / source of reference
PCOP35	Cu^r	DAC
PXjCu99	Cu^r	MNS
pXvCu	Cu^r	RES
pRK2073	ColEI replicon, Tra^+ Mob^+ Sp^+, helper plasmid	Turner et al., 1984

[a]BRL, Bethesda Research Laboratories, Gaithersburg; CK, H.C. Kistler, University of Florida, Gainesville, FL; DAC, D. A. Cooksey, University of California, Riverside, CA; DLC, D. L. Coplin, Ohio State University, Columbus, Ohio; MNS, M. N. Schroth, University of California, Berkeley, CA; RES, R. E. Stall, University of Florida, Gainesville, FL.

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BIOGRAPHICAL SKETCH

Huseyin Basim was born in Antalya, Turkey on June 1, 1966. Huseyin graduated from Ankara University in 1987 with a B.S. in plant protection. Huseyin enrolled in the graduate program of the same department in Ankara on September 1987. In Ankara, Huseyin worked on antagonistic effects of *Bacillus subtilis* isolates against important plant pathogenic fungi. Huseyin graduated with a M. S. degree in plant pathology from the University of Ankara on March 16, 1990. Huseyin enrolled the plant protection department in Akdeniz University, Antalya as a research and teaching assistant in same year. Huseyin carried out research projects on epidemics and control of *Erwinia amylovora* that causes fireblight on pear and apple plants in Southwest Turkey, and biological control of postharvest diseases on pears and apples by *Bacillus subtilis*. Huseyin got a scholarship from Akdeniz University for the graduate program at the University of Florida. On January 1992, Huseyin enrolled in the English Language Institute at the University of Florida to learn English. Huseyin was awarded a Jayne C. Harder Scholarship by the English Language Institute. On January 1993, Huseyin enrolled in the graduate program at the University of Florida, Department of Plant Pathology, with Dr. R. E. Stall as his advisor. Huseyin was awarded for The Francis Aloysius Wood Memorial Award in Plant Pathology in recognition of outstanding graduate student research in plant pathology at the College of Agriculture on March 22, 1996.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert E. Stall, Chair
Professor of Plant Pathology

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Jeffrey B. Jones
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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